# Studies On Pyruvate Decarboxylase-Catalysed Acyloin Formation And The Effects Of Surfactants On Lipase-Catalysed Hydrolysis Of Esters

By

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"Science moves, but slowly slowly, creeping on from point to point."

Alfred Lord Tennyson (1809-1892), from *The Lord of Burleigh* (1842).

"The great tragedy of science- the slaying of a beautiful hypothesis by an ugly fact."

T.H. Huxley (1825-1895), from *Biogenesis and Abiogenesis viii.*

Für mama und papa.

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**Declaration**

The work described in this thesis is original of the author, except where acknowledgement has been made to results and ideas published previously. The work was carried out at the Department of Chemistry, University of Warwick, between March 1989 and March 1992 and has not been submitted previously for a degree at any other institution.

The format of this thesis is based on that described in the Instructions for Authors (1991) for papers submitted to the Journal of the Chemical Society, Perkin Transactions.

Parts of the research described in this thesis have appeared in the scientific literature as follows:

Enzyme activities in crude porcine pancreatic lipase: enantioselectivity in hydrolysis of the diacetate of 2-phenylpropane-1,3-diol. S. Bornemann, D. H. G. Crout, H. Dalton and D. W. Hutchinson, *Biocatalysis*, 1992, **5**, 297.

**Summary**

The effect of surfactants on the hydrolysis of achiral and chiral substrates by crude and purified porcine pancreatic lipase (PPL; EC 3.1.1.3) has been studied. Rather than accelerating the reactions, surfactants slowed down ("inhibited") the reactions relative to the rate in the absence of surfactant, despite effective emulsification of the substrate. Surfactants varied in the extent to which the reaction was inhibited and inhibition occurred below the critical micelle concentration of surfactants. Inhibition was accompanied by a loss of enantioselectivity with the crude enzyme but not the purified enzyme, indicating the presence of more than one activity in the crude PPL preparation. In general, there would seem to be no advantage to be gained from the use of surfactants in the hydrolysis of compounds of low water solubility with lipolytic enzymes; the use of an immiscible cosolvent is more effective.

The pyruvate decarboxylase (EC 4.1.1.1) from *Zymomonas mobilis* strain CP4 ATCC 31821 was purified from recombinant *Escherichia coli* harbouring the plasmid pLOI295, which contained the gene coding for the enzyme. The purified recombinant enzyme catalysed acyloin condensations with a number of aldehyde acceptors. The substrate specificity of the *Zymomonas* enzyme was very similar to that observed with the enzyme from *Saccharomyces carlsbergensis*. However, the *Zymomonas* enzyme was found to catalyse the formation of acyloins from acetaldehyde at a rate four orders of magnitute greater than that observed with yeast enzyme. By comparing the stereochemistry of acyloin condensations catalysed by the *Zymomonas* and yeast enzymes, differences in the architecture of the active sites of these closely related enzymes have emerged.

## Abbreviations

ADP - adenosine diphosphate
AMP - adenosine monophosphate
ATP - adenosine triphosphate
b.p. - boiling point
bistrispropane - bis-(tris-(hydroxymethyl)-amino)-propane
CI - chemical ionization
CMC - critical micelle concentration
de - diastereomeric excess
DSS - sodium 2,2-dimethyl-2-silopentane-5-sulfonate
E - enantiomeric ratio
EC - Enzyme Commision classification number
ee - enantiomeric excess
$ee_p$ - product enantiomeric excess
$ee_s$ - substrate enantiomeric excess
EI - electron ionization
eq. - equivalents
FAD - flavin adenine dinucleotide
FPLC - fast protein liquid chromatography
GC - gas chromatography
h - hour
HETDP - 2-(1-hydroxyethyl)-thiamine diphosphate
HLB - hydrophile-lipophile balance number
HPLC - high performance liquid chromatography
IR - infra-red
lit. - literature reference
m.p. - melting point
min - minute
N - aggregation number
NAD - nicotinamide adenine dinucleotide
NADH - reduced nicotinamide adenine dinucleotide
NMR - nuclear magnetic resonance
No. - number
OD - optical density
PAC - phenylacetylcarbinol
PAGE - polyacrylamide gel electrophoresis
PDC - pyruvate decarboxylase
PDH - pyruvate dehydrogenase

PPL - porcine pancreatic lipase
ppm - parts per million
PVA - polyvinyl alcohol
rpm - revolutions per minute
SDS - sodium dodecyl sulfate
TDP - thiamine diphosphate
TLC - thin layer chromatography
Torr - (101325/760) N $m^{-2}$
Tris - tris-(hydroxymethyl)-aminomethane
UV - ultra violet
v/v - volume per volume
w/v - weight per volume
YPDC - yeast pyruvate decarboxylase
ZMPDC - *Zymomonas mobilis* pyruvate decarboxylase

All other surfactant and emulsifier abbreviations are listed in Figure 3.1.

## 1.1 General Introduction

The field of biotransformations is an important and rapidly expanding branch of synthetic organic chemistry. Biotransformations may be defined as the chemical modification of substrate molecules by biological catalysts. The practical application of biotransformations dates back to the production of ethanol and acetic acid from carbohydrates, by microbial fermentation, many thousands of years ago. More recently, the scope of this technology has been extended to include the production of non-natural products from non-natural substrates.[1]

There are many advantages that biotransformations have over conventional synthetic organic chemistry:[1]

- biotransformations are generally conducted in mild conditions (*e.g.* ambient temperature and physiological pH) and biocatalysts are generally very efficient catalysts and highly reaction specific; the possibility of unwanted side reactions, particularly of relatively labile substrates and products, are therefore greatly reduced;
- biocatalysts are often remarkably substrate tolerant; thus a single biocatalyst can be used to convert a whole series of substrates;
- the isolated enzyme or whole cell biocatalyst often can be produced cheaply and in large quantities by fermentation.

The concept of stereochemistry in biological processes goes back to Pasteur and van't Hoff-Le Bel, about a hundred years ago. In their memoirs,[2] Pasteur stated: "Most natural organic products, the essential products of life are asymmetric and possess such asymmetry that they are not superposable on their images... This establishes perhaps the only well marked line of demarcation that can at present be drawn between the chemistry of dead matter and the chemistry of living matter." Chirality, stereoselectivity and stereospecific production of chemicals are

characteristics of nature.[3] Biocatalysts catalyse reactions with complete regio-, diastereo- and enantiospecificity in almost every case.[4] This is arguably the most important property of biotransformations.[1] Therefore, there has been a surge of interest in biotransformations, over the past decade or so, for the production of enantiomerically pure compounds.

The production of enantiomerically pure compounds is becoming increasingly important as the drug, agrochemical and food regulatory authorities are putting more and more emphasis on the enantiomeric purity of new and existing drugs, agrochemicals and food additives.[5] There are many examples of the importance of stereochemistry for biological activity. D-Alanine, D-asparagine and D-isoleucine are sweet but the L-isomers are sweet, tasteless and bitter, respectively.[6] Perhaps the most poignant example is that of thalidomide. This sedative and mild-hypnotic drug was widely administered as a racemic compound before it was realised that it had teratogenic properties. Recent evidence suggests that it is the (-)-(*S*)-isomer that has the teratogenic activity, while the (+)-(*R*)-isomer has only the desired activities.[5]

There are several alternative strategies for enantioselective synthesis that have been well developed. The chiral pool is comprised of enantiomerically pure natural products, which can be used as chiral synthons. However, the chiral pool is finite. Chemical asymmetric synthesis and the resolution of stereoisomers by physical and chemical methods are also limited in scope. In the near future, biocatalysts offer many more opportunities for asymmetric synthesis and kinetic resolutions of chiral, *meso* and prochiral molecules.[1]

There are numerous reviews covering the field of biotransformations.[1,7-16] Biotransformations can be divided into those that are catalysed by whole cells and those that are catalysed by isolated enzymes. The multitude of enzymes present in

whole-cell catalysts make these catalysts difficult to characterise and manipulate. Therefore isolated enzymes have been studied more extensively than whole cells.

Enzyme have been classified by the type of reaction they catalyse using the Enzyme Commission numbering system:

EC 1. oxidoreductases
EC 2. transferases
EC 3. hydrolases
EC 4. lyases
EC 5. isomerases
EC 6. ligases

Not all of the enzyme classes have, so far, been exploited as biocatalysts. The majority of biotransformations have been restricted to oxidoreductase- and hydrolase-catalysed reactions.[1] In addition, the bulk of biotransformations have been carried out with very few biocatalysts.[1] Therefore, the breadth and number of applications of biotransformations has the potential of growing ever more rapidly.

Some of the limitations and perceived disadvantages of biotransformations[1,15] include:

- substrate and product inhibition
- low substrate solubility in aqueous environments
- inapplicability to some important types of reaction
- instability of biocatalysts
- the requirement of cofactors for certain biocatalysts
- the lack of predictive rules for the choice of biocatalyst
- the lack of fundamental enzymological knowledge

The studies described in this thesis address the lack of fundamental enzymological knowledge in 2-oxo-acid-lyase-catalysed (EC 4.1.1.1) biotransformations and the problem of low substrate solubility in aqueous environments in carboxylic ester hydrolase-catalysed (EC 3.1.1) biotransformations. Particular attention will be paid to enantioselectivity. The literature, relevant to these studies, will be reviewed in the appropriate chapters.

## 1.2 References


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# 2 Studies on Pyruvate Decarboxylase-Catalysed Acyloin Formation

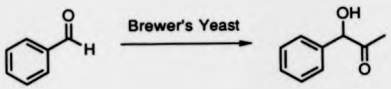
## 2.1 Introduction

**2.1.1** *Whole-cell-catalysed acyloin formation.*— One of the most basic biological acyloin products is acetoin (3-hydroxy-2-butanone). Acetoin is a valuable component in flavours and fragrances.[1,2] Its presence has been detected in numerous biological systems including many animal and plant tissues,[3] mammalian tumour cells,[4] bacterial cells,[5-10] fruit,[1,11] alcoholic beverages,[1,12,13] dairy products, cereals, vegetables, meat, honey and coffee.[1] This natural product was found to be optically active[3,9,14] and the stereochemistry of acetoin production will be discussed in terms of the enzymology of the systems in sections 2.1.4.4 and 2.1.4.6. Despite having been first discovered in the early part of the century, it is still not clear as to what its biological role is. It has been suggested that acetoin is produced from glucose metabolism *via* pyruvate either as an energy store,[2,15] as an alternative to the production of relatively more toxic acidic[16] or aldehydic[4] metabolites, as a regulator of intermediary metabolism[4] or as a redox "buffer".[17]

The first reports of unnatural biological acyloin products (reviewed by Csuk and Glaenzer)[18] were over seventy years ago for the transformation of benzaldehyde[19-21] to phenylacetylcarbinol (PAC, 1-hydroxy-1-phenyl-2-propanone) using fermenting brewer's yeast (Figure 2.1). Neuberg and Hirsch[19] also showed that a yeast cell-free system retained the ability to convert benzaldehyde into PAC using pyruvate as a cosubstrate. Acetaldehyde (ethanal) was not found to be a suitable cosubstrate as benzoic acid rather than PAC was produced. The PAC obtained was optically active, the

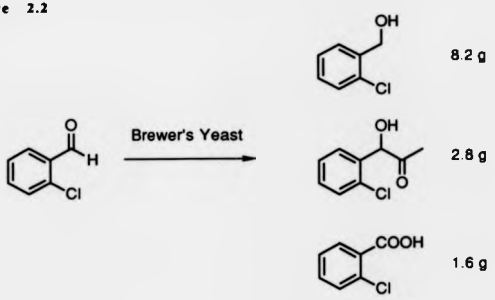
phenylhydrazone having a substantial optical rotation. However, the optical purity and absolute configuration were not determined.

**Figure 2.1**




Later, in 1921, Neuberg and Liebermann[22] showed that 2-chlorobenzaldehyde could also be converted into the corresponding optically active acyloin. Large amounts of 2-chlorobenzylalcohol and smaller amounts of 2-chlorobenzoic acid were also obtained (Figure 2.2). The alcohol and acid corresponding to the aldehyde substrate have since been found to be common by-products of acyloin formation.[23-27] Neuberg and Liebermann[22] also reported the formation of an acyloin from anisaldehyde.

**Figure 2.2**




In 1922 a further transformation product of benzaldehyde, 1-phenyl-1,2-propanedione, was reported.[28] In 1926, Behrens and Ivanoff[29] described the formation of acetylcarbinols from 2- an 4-tolualdehydes (Figure 2.3). They also reported the formation of the corresponding diol from 4-tolualdehyde (Figure 2.4).

**Figure 2.3**

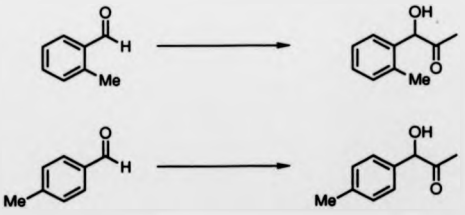


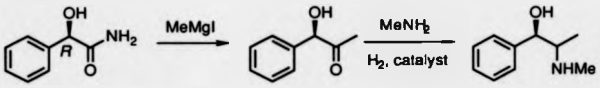
**Figure 2.4**


In 1930, PAC found its industrial application[30] in the production of (-)-ephedrine [(1*R*, 2*S*)-(-)-2-(*N*-methylamino)-1-phenyl-1-propanol; Figure 2.5]. This was one of the first industrial processes combining both microbial and chemical synthesis[31-33] and this process is still used today. Ephedrine is a natural plant product of the genus *Ephedra*[34] and has been used for at least 5000 years in China in the treatment of disorders of the respiratory tract.[35] Ephedrine was first used as a sympathomimetic drug in western medicine in 1923. The nasal mucosal vasoconstrictor activity of ephedrine has proved useful in the treatment of allergic rhinitis and hay fever. Ephedrine has also been used as a general vasoconstrictor in the treatment of vascular collapse caused by allergic and anaphylactic states. It is also a bronchial dilator and has been used in the treatment of asthma. (1*R*,2*S*)-(-)-Ephedrine is the most pharmacologically potent ephedrine stereoisomer. Several analogues of ephedrine, including the dimethylamino derivative, appear to exhibit similar sympathomimetic activities to ephedrine, but are in general less potent vasoconstrictors and more potent bronchial dilators.

**Figure 2.5**

The reductive amination of PAC to (-)-ephedrine proceeds in yields of approximately 20%, to give, apparently, an optically pure product after crystallisation of the corresponding hydrochloride.[30] The reduction would therefore appear to occur with a high degree of diastereoselectivity. X-Ray crystallographic studies of (-)-ephedrine[36] led to the unambiguous assignment of microbially produced (-)-PAC as having an *(R)* absolute configuration.[37] In addition, (R)-(-)-mandelic acid amide has been converted to (-)-PAC, which in turn has been converted into (-)-ephedrine (Figure 2.6).[38] Thus, there is no doubt about the absolute configuration of (-)-PAC produced by yeast.

**Figure 2.6**




(-) (-) (-)

In 1963, Becvarova and Hanc broadened the scope of PAC production by showing that genera other than *Saccharomyces* were capable of catalysing PAC production from benzaldehyde (Table 2.1).[39] However, the best organism was *S. carlsbergensis*, which was able to achieve a 70% conversion of benzaldehyde to PAC. PAC was estimated both polarographically and polarimetrically. The difference between these two methods of determination is a function of optical purity. Calculated enantiomeric excesses (not given by the original authors and which must be considered only partly reliable in view of the fact that contaminating optically active substances may have affected the polarimetric determination) are given in Table 2.1.

**Table 2.1** The optical purity of PAC produced from benzaldehyde by yeasts[39]

| Species | ee (%) |
|---|---|
| *Hansenula anomala* | 92 |
| *Brettanomyces vini* | 87 |
| *S. carlsbergensis* | 81 |
| *S. cerevisiae* | 65 |
| *S. ellipsoideus* | 89 |
| *Torula utilis* | 71 |

Acyloins, including PAC, are prone to racemisation, particularly in the presence of base.[28] This process can also lead to isomerisation of PAC to benzoylmethylcarbinol (2-hydroxy-1-phenyl-1-propanone; Figure 2.7). Favorsky[40] reported that this isomerisation could be catalysed by brewer's yeast. However, the experimental evidence for this transformation was not conclusive. Only one further report of the production of benzoylmethylcarbinol has appeared and no experimental evidence was given.[27] Neuberg and Ohle[28] did isolate 1-phenyl-1,2-propanedione as a by-product of PAC production and reduction of this could give benzoylmethylcarbinol. Evidence that this might occur is contained in a recent report[41] that the diketone is reduced by yeast to a mixture of (*R*)-PAC and (*S*)-benzoylmethylcarbinol, and that these are reduced to (1*R*,2*S*)-1-phenyl-1,2-propanediol (Figure 2.8).

**Figure 2.7**

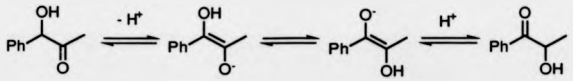
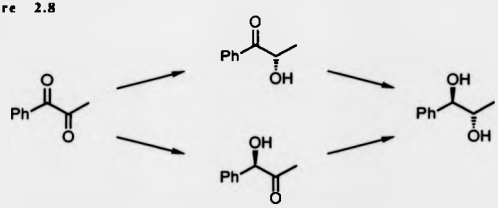



**Figure 2.8**



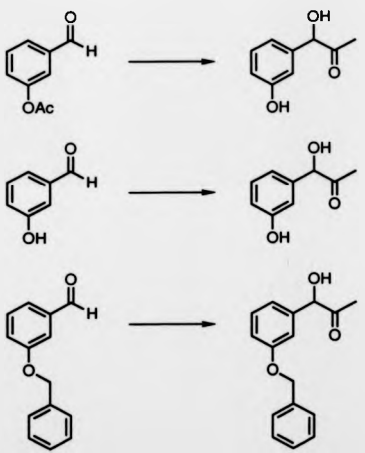

Becvarova and Hanc also emphasised two further factors that were important in optimising PAC production.[39] Firstly, the addition of acetaldehyde was considered to be beneficial. As a scavenger of dehydrogenase activity, acetaldehyde appeared to reduce the formation of benzyl alcohol. Secondly, the controlled addition of benzaldehyde was considered to be important as high levels of this somewhat toxic substrate led to the cessation of the fermentation of the microorganism and PAC production. *Hansenula anomala* appeared to be the least prone to benzaldehyde toxicity.

More recently, Gupta *et al.* and Cardillo *et al.* observed PAC production from benzaldehyde with most of the *Saccharomyces* species tested.[23,42] Netrval and Vojtisek screened 38 yeast species for PAC production.[43] They concluded that yeasts of the genera *Saccharomyces* and *Candida* were generally better PAC producers than those of the genus *Pichia*. *Saccharomyces carlsbergensis* strain "Budvar" produced the most PAC and production was

slightly lower in four strains of the genera *Saccharomyces*, *Candida* and *Hansenula*. Non-producers included *Saccharomycodes luwigii* and *Schizosaccharomyces japonicus*.

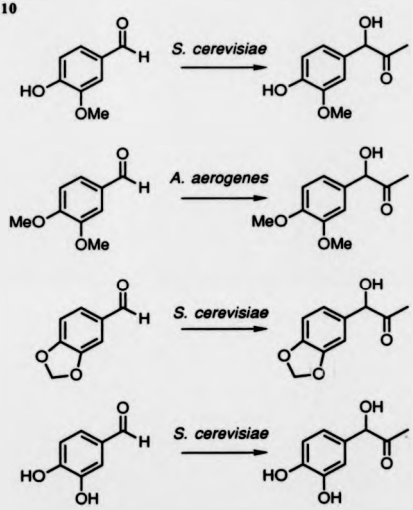
Further reports in the early 1930s extended the scope of the *Saccharomyces cerevisiae*-catalysed acyloin condensations to include a variety of substituted aromatic aldehydes (Figure 2.9).[44,45] For example, 3-acetoxybenzaldehyde yielded the corresponding acetylcarbinol with concomitant hydrolysis of the acetyl ester.

**Figure 2.9**



In addition, two 1965 patents described the the conversions of 3,4-disubstituted benzaldehydes (Figure 2.10).[46,47] It is interesting that the conversion of veratraldehyde (3,4-dihydroxybenzaldehyde) was catalysed by *Aerobacter aerogenes*, a prokaryotic bacterium. The acetylcarbinol products were converted into 3,4-substituted L-2-methylphenylalanine derivatives, claimed to be valuable agents for treating hypertension.

**Figure 2.10**




In 1968 the range of suitable aldehydes was extended (Figure 2.11).[48] Conversions of these aldehydes to acyloins was reported to be between 35 and 50%. Of particular interest is the apparent formation of acyloins from

aliphatic aldehydes other than propanal and acetaldehyde. This appears to be the only report of long chain aliphatic aldehydes being substrates for acyloin formation. A number of additional aldehydes also appeared to give rise to acyloins (Figure 2.12).[48] It was presumed that some aldehydes did not give rise to acyloins because of their high toxicity (Figure 2.13).[48]

**Figure 2.11**

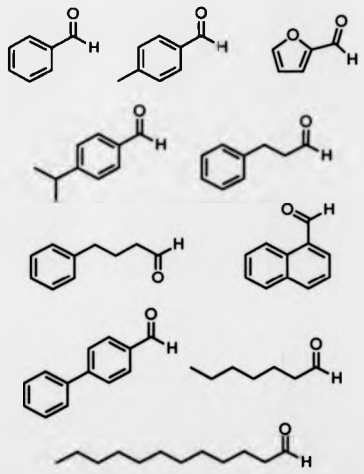


**Figure 2.12**

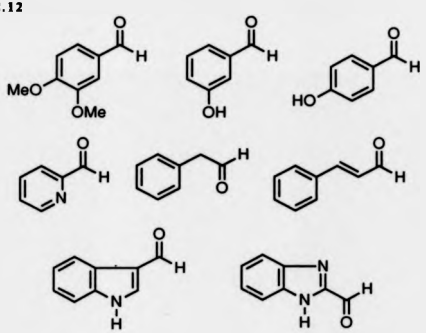
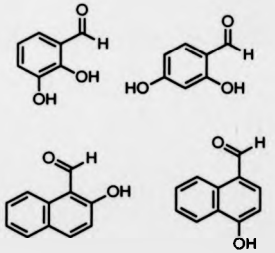



**Figure 2.13**



Fuganti and Grasselli isolated 1-phenyl-1,2-propanediol rather than PAC from the transformation of benzaldehyde (Figure 2.14) and determined the absolute configuration as being (1*R*,2*S*) by polarimetric comparison of the dibenzoyl derivative with authentic material.[49] The diol was obtained in high optical purity [> 95% enantiomeric excess (ee)] and the diastereomeric excess (de) was determined to be 80% *erythro* by gas chromatographic analysis of the corresponding acetonide and comparison with authentic samples. Recently, Ohta *et al.* isolated the diols from a series of substituted benzaldehydes (Figure 2.14 and Table 2.2).[50] They determined the de and ee by similar methods except that the optical purity was determined by chiral high performance liquid chromatography (HPLC), rather than polarimetry, of the dibenzoates.

**Figure 2.14**

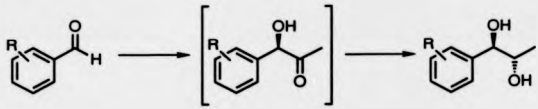


**Table 2.2** The conversion of substituted benzaldehydes to (1*R*,2*S*)-diols[50]

| R[a] | Yield (%) | ee (%) | de (%) |
|---|---|---|---|
| H | 30 | 97 | 94 |
| 2-Me | 7 | 97 | 98 |
| 4-Me | 28 | 99 | 96 |
| 2-Cl | 32 | 97 | 98 |
| 4-Cl | 27 | 98 | 96 |
| 4-OMe | 22 | 97 | 94 |
| 2-$CF_3$ | 0 | n.d.[b] | n.d.[b] |
| 3-$CF_3$ | 0 | n.d.[b] | n.d.[b] |
| 4-$CF_3$ | 0 | n.d.[b] | n.d.[b] |
| 2-F | 30 | 97 | 98 |
| 3-F | 31 | 97 | 86 |
| 4-F | 26 | 97 | 94 |
| 4-$NO_2$ | 14 | n.d.[b] | n.d.[b] |

[a]See Figure 2.14
[b]Not determined

More recently, Long's group identified similarly substituted aromatic aldehyde substrates (Figure 2.15 and Table 2.3).[25,26] In this case the acetylcarbinols were isolated. It was noted that the reactivity of the 2-substituted benzaldehydes was lower than that for the 4-substituted derivatives. The low reactivity of the α,α,α-trifluorotolualdehydes should be compared with their complete lack of reactivity reported by Ohta *et al.* in the formation of the corresponding diols.[50]

**Figure 2.15**


**Table 2.3** The conversion of substituted benzaldehydes to acetylcarbinols by *S. cerevisiae*[25,26]

| R[a] | Acetylcarbinol[b]/mg cm$^{-3}$ |
|---|---|
| H | 10.1- 10.2 |
| 2-Me | 2.0- 2.5 |
| 3-Me | 5.2- 6.2 |
| 4-Me | 5.4- 6.4 |
| 2-Cl | 0.6- 0.7 |
| 3-Cl | 2.1- 3.2 |
| 4-Cl | 6.5- 8.0 |
| 2-OMe | 0.8- 0.9 |
| 3-OMe | 4.5- 5.7 |
| 4-OMe | 1.2- 3.4 |
| 2-$CF_3$ | 0.2- 0.3 |
| 3-$CF_3$ | 0.3- 0.4 |
| 4-$CF_3$ | 0.5- 0.8 |
| 4-$NO_2$ | 0.0 |

[a]See Figure 2.15

[b]Aldehyde was added in portions, the total added being equivalent to a concentration of 10 mg cm$^{-3}$.

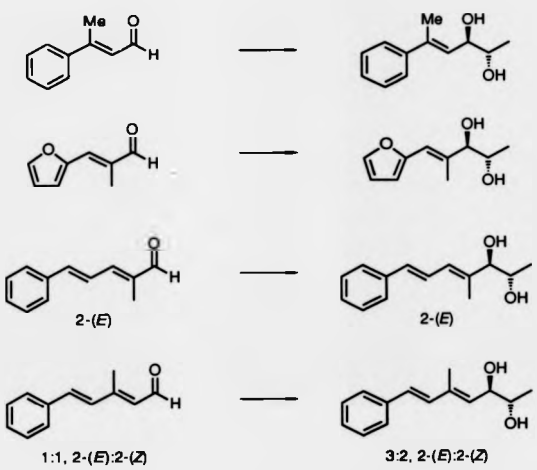
Fuganti and his colleagues have investigated the formation of acyloins from α,β-unstaturated aldehydes. The conversion of cinnamaldehyde, 2-bromocinnamaldehyde and 2-methylcinnamaldehyde[49] to the corresponding (*R*)-acetylcarbinols[51] were initially described. However, the acetylcarbinols were not the major products to be isolated. It appeared that the acyloin products were subsequently reduced to diols *in situ* (Figure 2.16). Another competing reaction was the reduction of the aldehyde substrates to alcohols.[49] It is of interest however, that the corresponding allylic alcohols could be used as substrates because the alcohols could, presumably, be oxidised *in situ* to the aldehydes.[49] By comparison with known compounds, the absolute configuration of the diols were determined to be (2*S*,3*R*).[52] Therefore, Fuganti concluded that there is *si*-face attack of the aldehyde substrate to give the (*R*)-acetylcarbinol which, in turn, undergoes *re*-face reduction to give the *erythro*-diol.[53] The corresponding 2-butyl and 2-propyl compounds were not substrates.[54] The 2-substituent therefore played a major role in the reactivity of the cinnamaldehydes.

**Figure 2.16**


R = H, Me, Br

More recently, several other α,β-unstaturated aldehydes have been shown to be converted to the corresponding diols (Figure 2.17).[54-56] The apparent preference for the 2-(*E*) isomer of 3-methyl-5-phenyl-2,4-pentandienal to be converted illustrated the importance of the geometry of the 2-carbon double bond in this biotransformation.

Figure 2.17




Fuganti and his colleagues have exploited the formation of these *erythro*-diols in the chiral synthesis of a wide variety of compounds of interest,[57,58] including the enantiomeric forms of 4-hexanolide (γ-carprolactone),[59] D-(-)-*allo*-muscarine,[60] deoxysugars,[52,54,61-64] (+)-*exo*-brevicomin,[64] octanolide,[64] L-olivomycose,[54] pheromones,[65,66] (-)-frontalin,[67] an α-tocopherol (vitamin E) intermediate,[56] and an $LTB_4$ (chemotactic factor) intermediate.[68]

All of the acyloins discussed so far have been acetylcarbinols, where the acetyl group is derived from pyruvate. The substrate specificity of the acceptor aldehyde has been demonstrated to be quite broad, particularly with substituted aromatic and α,β-unsaturated aldehydes. However, the substrate specificity of the 2-oxo (α-keto) acid donor is less well understood. 3-Hydroxy-2-pentanone has been detected in yeast fermentations and wine.[12,13] Indeed, Suomalainen and Linnahalme showed that the addition of pyruvate and 2-oxobutanoic acid to fermenting brewer's yeast gave predominantly 3-hydroxy-2-pentanone and smaller amounts of 2-hydroxy-3-pentanone.[69] They also observed the formation of propionin (4-hydroxy-3-hexanone) when 2-oxobutanoic acid was added without pyruvate. It was later noted that dissociated 2-oxo acids were not able to penetrate the plasma membrane of yeast and that either an acidic fermentation medium or aerobic fermentation conditions increased the cellular uptake of these acids.[70] The production of an acyloin has been detected on addition of propanal, although no experimental details were given.[26] It is assumed that this product was 3-hydroxy-2-pentanone.

Fuganti and colleagues showed that beside pyruvate, 2-oxobutanoate and 2-oxopentanoate could act as donors with washed cells of baker's yeast, although the effectiveness of 2-oxopentanoate seemed to be dependent on the nature of the acceptor aldehyde (Figure 2.18).[55] The products from benzaldehyde were obtained with greater than 95% enantiomeric excess (ee). Conversion of the acyloin product **1** into the corresponding (2*S*,3*R*)-diol **2** in an optically pure form by fermenting baker's yeast suggests that this product was also formed in a high degree of optical purity.

Abraham and Stumpf described 1,2-dihydoxypropyl compounds obtained from isoprenoids *via* the terminal terpendiols by *Corynespora cassiicola* or *Diplodia gossypina*.[71] The 1,1-dimethyl-2,3-diols were found to be cleaved

presumably to an acetone moiety and an aldehyde, which was prolonged by a $C_2$ unit. The formation of the intermediary aldehyde as a starting structure for subsequent acyloin condensation was confirmed by isolation of the respective primary alcohol.

Kawabata *et al.* isolated (2*S*,3*R*)-*trans*-4-*trans*-6-octadien-2,3-diol as a product of sorbic acid metabolism using *Mucor* sp.[72] It was assumed that the intermediate in this reaction was sorbaldehyde. Very recently, Stumpf and Kieslich studied acyloin condensations of acyclic unsaturated aldehydes by *Mucor circinelloides* (Figure 2.19).[73] The acyloins were reduced *in situ* to (2*S*,3*R*)-diols. Reduction of the aldehyde group, including hydrogenation of the conjugated C-C double bond, hydroxylation of these alcohols and of the formed diols and some cyclisations were also found as side reactions.

**Figure 2.18**

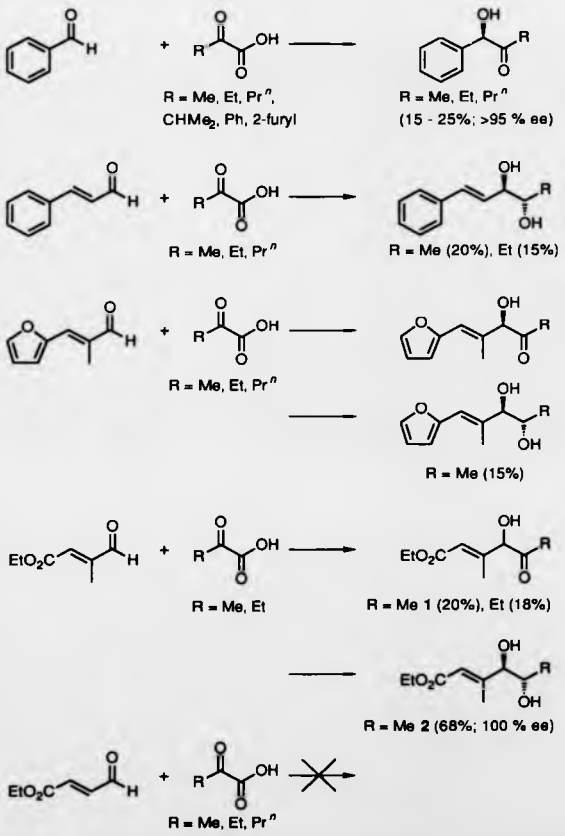
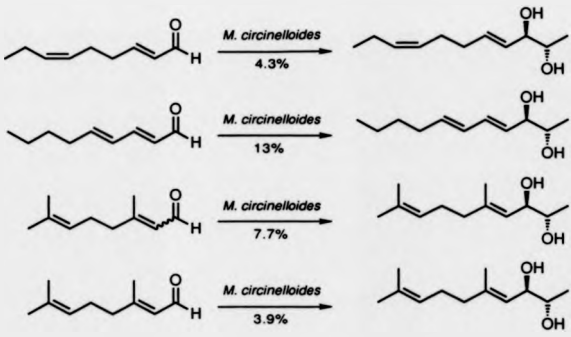

R = Me, Et, Pr$^n$, CHMe$_2$, Ph, 2-furyl
R = Me, Et, Pr$^n$ (15 - 25%; >95 % ee)
R = Me, Et, Pr$^n$
R = Me (20%), Et (15%)
R = Me, Et, Pr$^n$
R = Me (15%)
R = Me, Et
R = Me 1 (20%), Et (18%)
R = Me 2 (68%; 100 % ee)
R = Me, Et, Pr$^n$

**Figure 2.19**




Cardillo *et al.* have made the most recent contribution to the study of yeast catalysed acyloin condensations.[23] They screened a number of yeasts and bacteria, and found that *Saccharomyces fermentati* and *S. delbrueckii* produced almost twice as much 1-phenyl-1,2-propanediol than did *S. cerevisiae* under standard reaction conditions. However, the ability to condense other aldehydes, such as cinnamaldehyde, 2-methylcinnamaldehyde and 2-furaldehyde, was only significant with *S. cerevisiae*. Similarly, *S. cerevisiae* was the only microorganism capable of condensing 2-oxo acids other than pyruvate, although no experimental detail was given.

**2.1.1.1** *Physiological factors in PAC production.* Fuganti *et al.* also observed the beneficial effect of adding acetaldehyde in the production of acyloins.[53] Interestingly, the addition of propanal and butanal did not result in the production of the corresponding propanoyl and butanoyl carbinols. The role

of added acetaldehyde was probably not as a substitute for pyruvate as a cosubstrate in the acyloin condensations.

Since Neuberg and Ohle[28] first observed the production of benzyl alcohol as a by-product in the formation of PAC, much attention has been paid to reducing the extent of this wasteful side-reaction. Smith and Hedlin approached this problem by investigating the effect of dehydrogenase inhibitors.[74] Some nicotinamide adenine dinucleotide (NAD) analogues inhibited benzyl alcohol formation with a concomitant increase in PAC production. These inhibitors were 3-acetylpyridine, pyridine-3-sulfonic acid, α-picolinic acid, pyrazinamide, nicotinamide, isonicotinamide and isonicotinic acid.

The favoured approach to minimising alcohol production has been to add an electron acceptor,[75] such as acetaldehyde,[39] rather than to attempt to inhibit dehydrogenase activity.[74] This finding explains the beneficial effect of added acetaldehyde as observed by Fuganti *et al.*[53]

Ward *et al.* have observed the reduction of aromatic aldehydes by yeast alcohol dehydrogenase.[26] Nikolova and Ward used *S. cerevisiae* mutants, lacking the dominant alcohol dehydrogenase isoenzymes I, II and III, to produce PAC.[27] No significant reduction in the formation of benzyl alcohol was observed, suggesting that the availability of reduced nicotinamide adenine dinucleotide (NADH) was rate limiting in the reduction of benzaldehyde rather than alcohol dehydrogenase activity. However, the presence of low levels of additional isoenzymes was detected and the presence of other activities capable of reducing benzaldehyde was not ruled out. A common observation has been the reduced levels of benzyl alcohol production when pyruvate is added as the cosubstrate rather than hexose sugars, such as glucose.[25] This is consistent with the view that NADH

concentration is rate-limiting in the reduction of benzaldehyde because NADH is formed in the normal glycolytic metabolism of glucose to pyruvate.

The production of 1-phenyl-1,2-propanedione and benzoic acid as by-products in the formation of PAC is much less significant than the production of benzyl alcohol.[28] Therefore, there has been no attempt to minimise these side-reactions. There is one report of the production of *trans*-cinnamaldehyde from benzaldehyde.[76] This curious by-product must have undergone several distinct transformations and further studies are clearly required in order to account for this finding.

A constant theme in more recent investigations has been the need to control benzaldehyde addition.[24-26,39,42,76] Benzaldehyde appears to reduce cell viability and the rate of PAC formation at concentrations much above 2 g $dm^{-3}$ causes complete inhibition of the formation of PAC at a concentration of 8 g $dm^{-3}$. Therefore, the addition of benzaldehyde must be controlled. Four batch-wise additions of this substrate such that the benzaldehyde concentration does not exceed about 1 g $dm^{-3}$ appears to be the most common solution to this problem. The toxicity of other aldehyde substrates has been observed. Examples include salicylaldehyde,[48,77] which does not appear to form acyloins,[26] and heptanal.[48]

Another approach to the problem of benzaldehyde toxicity has been the isolation of microbial mutants, resistant to benzaldehyde. Ellaiah and Krishna isolated a *S. cerevisiae* mutant, after chemical mutagenesis, that was capable of producing 12% more PAC than the wild-type strain.[78] Strains selected for benzaldehyde (8 g $dm^{-3}$) and PAC (9 g $dm^{-3}$) resistance produced 10 and 6% more PAC, respectively. Similar studies have been described, where *S. cerevisiae* and *Candida flareri* strains were mutated and selected for

acetaldehyde, ephedrine and 1-phenyl-1,2-propanedione resistance.[79] Some mutant strains were isolated that were capable of elevated PAC production.

Mahmoud *et al.* investigated two further approaches to this problem. Firstly, they immobilised *S. cerevisiae* cells with alginate beads and found that in both batch cultures and semicontinuous fermentations benzaldehyde toxicity was reduced, resulting in higher PAC yields.[34,80] Secondly, the addition of β-cyclodextrin to the fermentation medium reduced benzaldehyde toxicity.[81] This effect was attributed to the formation of an inclusion complex formed by benzaldehyde and the β-cyclodextrin, which reduced the free concentration of benzaldehyde. The benzaldehyde complex dissociated slowly to allow the formation of PAC.

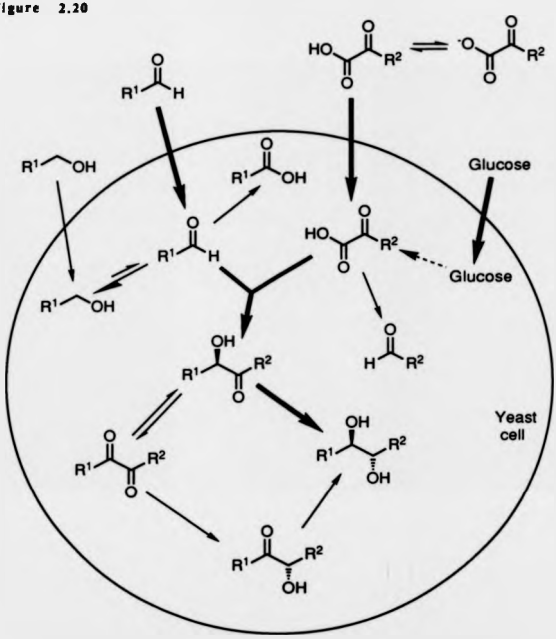
Several other factors have been found to be important in the production of PAC. The pH of the fermentation medium can affect the production of PAC,[25,26,42,78] particularly in terms of the rate of the further reductive transformation of PAC to 1-phenyl-1,2-propanediol.[50] PAC production is favoured in a medium of pH between 4.0 and 5.0.[25,26,42,50,78] The production of the diol predominated at neutral and basic pH, with an optimum of between pH 8 and 9.[50] Interestingly, acetoin production from pyruvate by *Lactobacillus plantarum* was found to be optimal at pH 4.5 (95% conversion) with a marked decrease in conversion at pH 5.0 (46%).[6]

Another important factor is the aeration of the fermentation medium.[43,76,82-84] Ellaiah and Krishna[83] found that PAC production by *S. cerevisiae* reached an optimum rate at an oxygen transfer rate of 2.35 mm $dm^{-3}$ $h^{-1}$. Argarwal *et al*[84] found that the dissolved oxygen level at 30 °C needed to be in the range of 75-85% for efficient PAC production.

A further physiological factor of significance was found to be cell density. It was reported that the lower the cell density, the greater the specific rate of PAC production (mmol g. dry cells$^{-1}$ h$^{-1}$).[84] It was suggested that this was related to oxygen limitation at high cell densities. In addition, cell age was found to affect the specific activity. A cell age of between 15 and 24 hours was optimal. Similarly, in continuous fermentations, the greater the rate of cell dilution, the greater was found to be the specific rate of PAC production by cells harvested from the continuous culture.[85]

**2.1.1.2** *Summary*. The whole-cell mediated acyloin condensation reaction can be summarised as shown in Figure 2.20. This scheme may not show all of the possible side reactions, but the main reactions are highlighted in bold type. The main by-products are, in most cases, formed by redox reactions which are in principle reversible reactions. The most important point that this scheme illustrates is the abundance of side reactions that can occur in acyloin condensations. Thus a quantitative yield of acyloin is never achieved. This problem can be avoided by using the isolated enzyme(s) responsible for acyloin condensations. The enzymology of this system will be discussed below.

Figure 2.20



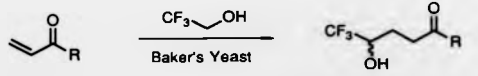

**2.1.2** *Michael Additions Involving Acyl Anion Equivalents.*— As a final example of the versatility of the yeast system, the transformation shown in Figure 2.21 should be cited.[86] Formation of the products can be interpreted in terms of the oxidation of trifluoroethanol to fluoral, formation of "active fluoral" and Michael addition of this to α,β-unsaturated carbonyl compounds. However, since free acetaldehyde has not been found to be an effective donor

in acyloin condensations catalysed by yeasts, other interpretations of the mechanism of these transformations should be considered. Some of the ester products subsequently formed lactones *in situ*.

**2.1.3** *Chemical synthesis of acyloins.*— There are several alternative chemical synthetic strategies for PAC formation. One of the first was discussed above (Figure 2.6).[38] (*R*)-Mandelic acid amide was converted to (*R*)-PAC, using MeMgI, in about 70% yield. The optical rotation of PAC was about two thirds of that obtained from the yeast biotransformation,[28] indicating that this chemical method produces PAC with an optical purity of about 66% ee.

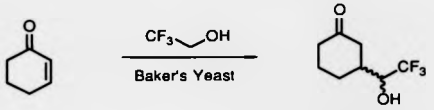
There are several examples of the chemical synthesis of racemic PAC using either (1-alkyloxyvinyl)lithium or (1-alkylthiovinyl)lithium as a reagent for nucleophilic acetylation (Figure 2.22).[87-90] In an alternative strategy, 1-phenyl-1,2-propanedione was reduced by aqueous $TiCl_3$ to PAC (Figure 2.23).[91] An interlamellar montmorillonite-silylamine-palladium(II) catalyst was used to the same effect.[92]

**Figure 2.21**

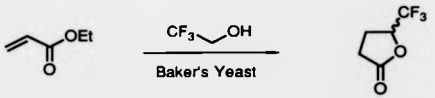



R = Me, Et

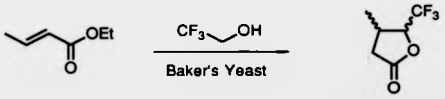
R = Me (26% yield; 93 % ee),
Et (41% yield; 91 % ee)



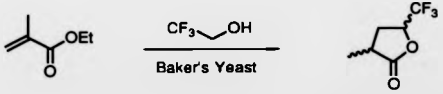

38% yield; 84 % de




47% yield; 79 % ee




43% yield; 72 % de




47% yield; 66 % de

**Figure 2.22**

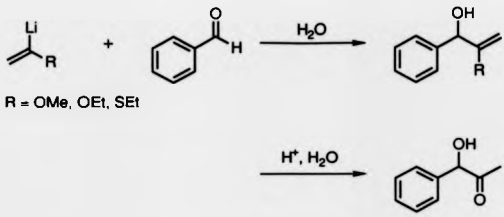
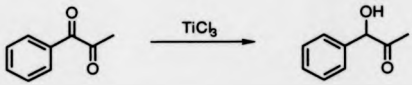
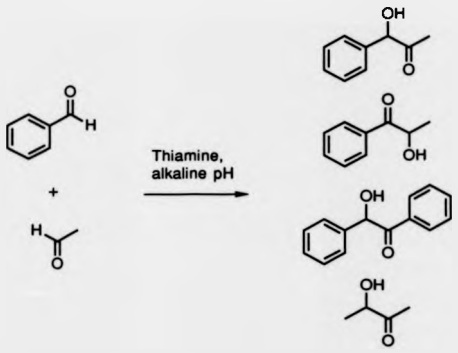



**Figure 2.23**




Mizuhara and Handler were the first to describe the value of thiamine as a catalyst in the synthesis of acetoin from acetaldehyde and pyruvate.[93] In an analogous reaction, 5-(2-hydroxyethyl)-3,4-dimethyl-1,3-thiazolium iodide can catalyse the formation of PAC from acetaldehyde and benzaldehyde in alkaline conditions.[94] The yield of PAC is rather low as acetoin, benzoylmethylcarbinol and benzoin (2-hydroxy-1,2-diphenylethanone) are also formed in this reaction (Figure 2.24).

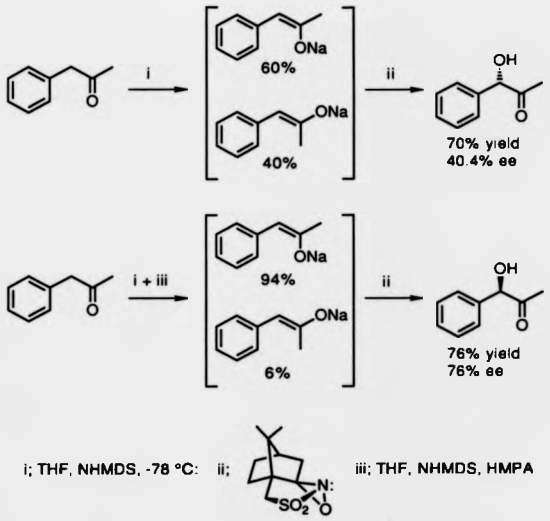
**Figure 2.24**




Although the above syntheses of racemic PAC are useful, they have no practical value in the synthesis of (-)-ephedrine.

A recent publication describes the asymmetric oxidation of ketone enolates, generated with sodium bis(trimethylsilyl)amide (NHMDS), using (+)-(camphorylsulfonyl)oxaziridine (Figure 2.25).[95] The presence of hexamethylphosphoramide (HMPA) altered the absolute configuration of the predominant product. However, in neither case was the PAC optically pure.

**Figure 2.25**



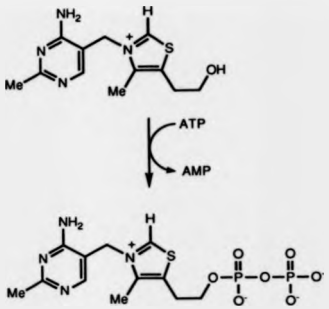

Although chemical syntheses of PAC have been developed, none appear to produce PAC of high optical purity and therefore the yeast-catalysed biotransformation remains the favoured method.

**2.1.4** *Enzymology of acyloin formation.*— **2.1.4.1** *Historical perspectives.* Progress with the production of PAC and analogues clearly depended on an understanding of the enzymology of this reaction. Neuberg had attributed PAC formation to the action of a "carboligase" [also known as α-carboxylase, pyruvate decarboxylase (PDC) and 2-oxo-acid-lyase, EC 4.1.1.1],[19] which was first described by Neuberg and Karczag in 1911 as the activity responsible

for the decarboxylation of pyruvate to acetaldehyde.[96] Neuberg had observed the formation of PAC from benzaldehyde and pyruvate in yeast cell-free systems.[19] Other workers had also observed such activity in cell-free systems and full activity was evident only when thiamine diphosphate (TDP, also known as thiamine pyrophosphate and cocarboxylase) and magnesium ions were added.[24,77] Smith and Hedlin found that the addition of coenzyme A and NAD were also necessary for maximum reaction rates.[97] They proposed an enzyme mechanism based on the formation of acetyl coenzyme A or acetyl phosphate from pyruvate. This work was carried out in the early 1950s, before the biological importance of TDP had been recognised.[98]

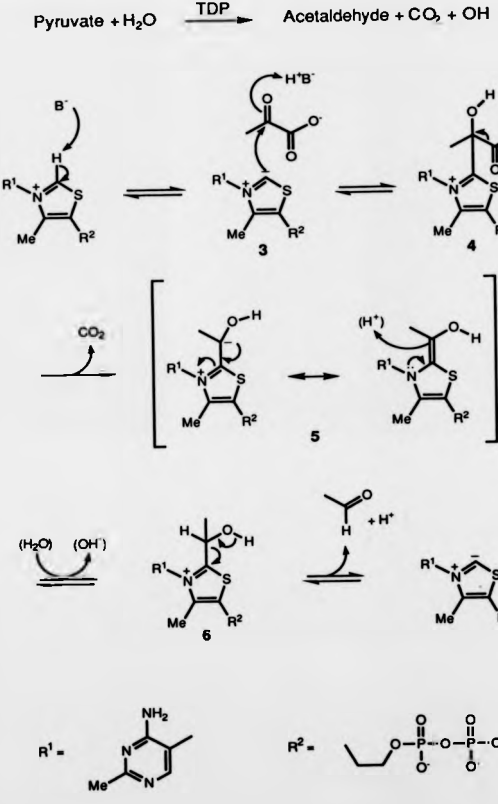
Since TDP was first described by Lohmann and Schuster in 1937,[99] it was recognised to be an essential coenzyme of many enzymes well before its precise role was understood. Thiamine is known, because of its dietary requirement,[100] as vitamin $B_1$ and TDP is formed in biological systems (Figure 2.26) from thiamine and ATP (adenosine triphosphate).[101] TDP serves a number of essential metabolic functions and its deficiency leads to imbalances in carbohydrate status and a number of diseases.[102]

**Figure 2.26**




**2.1.4.2** *Mechanistic aspects.* Breslow first proposed the mechanism by which TDP and thiamine catalyse biologically relevant reactions[103] after the discovery that thiamine itself catalysed reactions analogous to those of PDC.[93] A crucial step in TDP mediated catalysis is the formation of the ylide **3** by deprotonation of the carbon at the 2 position of the thiazolium ring.[103] Taking PDC as an example, the ylide **3** is able to attack the carbonyl carbon atom of 2-oxo acids, such as pyruvate (Figure 2.27). The resulting adduct, known as 2-(2-lactyl)-TDP **4**, undergoes decarboxylation to form the 2-(1-hydroxyethyl)-TDP zwitterion **5** (known as the HETDP carbanion), which is also known as "active acetaldehyde". The thiazolium ring serves as an electron sink for decarboxylation as the HETDP carbanion is resonance stabilised by the delocalisation of the carbanion negative charge to form the enamine.

**Figure 2.27**


Pyruvate + $H_2O$
TDP
Acetaldehyde + $CO_2$ + OH
$H^+B^-$
$B^-$
3
4
$CO_2$
5
$(H_2O)$
$(OH^-)$
6
$+H^+$
$R^1$ =
$R^2$ =

In PDC, the HETDP carbanion is protonated to form HETDP **6**. Thus, the formation of the HETDP carbanion effectively allows PDC to perform an electrophilic substitution reaction of a proton for carbon dioxide at an acyl carbon atom which, under normal circumstances, would be prone to nucleophilic substitution.[104] Although both lactyl-TDP[104] **4** and HETDP[105] **6** have been synthesised chemically, only HETDP has been successfully isolated from enzymic reaction systems,[105-109] as a stable reaction intermediate, because lactyl-TDP was found to undergo rapid and spontaneous decarboxylation.[104] Finally, HETDP **6** dissociates to liberate acetaldehyde and regenerate the coenzyme. Direct evidence of this last step comes from the observations of Krampitz *et al.*[105] HETDP was prepared chemically and was shown to be converted into acetaldehyde and TDP by the apoenzyme of wheat germ PDC.

All of the steps are essentially reversible except for the decarboxylation step. Model studies of the reverse reaction, from acetaldehyde to pyruvate, using thiamine as a catalyst have shown that carboxylation occurs only at high carbon dioxide pressures (50 atm).[110] Thus, in the normal physiological PDC-catalysed reaction, pyruvate is irreversibly decarboxylated to form acetaldehyde and carbon dioxide, with the concomitant deprotonation of a water molecule to form a hydroxide anion.

The isolation of HETDP[105-108] would suggest that one (or more) of the post-decarboxylation steps is rate limiting. Numerous studies of kinetic isotope effects (for example[111-116]) have been reported. A discussion of these studies is beyond the scope of the present investigation. However, the formation of lactyl-TDP appears to be, at least in part, rate determining

Other TDP-dependent enzymes have similar mechanisms, especially with regard to the formation of the HETDP carbanion as an intermediate. In

pyruvate oxidase (cytochrome) (EC 1.2.2.2), the HETDP carbanion is oxidised to acetyl-TDP, which is hydrolysed to liberate acetate (Figure 2.28). This oxidation step requires the cofactor ferricytochrome *b*1. The E1 component (EC 1.2.4.1) of the pyruvate dehydrogenase (PDH) complex performs a similar function to pyruvate oxidase, in that pyruvate is oxidatively decarboxylated to form acetyl-TDP,[117] which subsequently acetylates PDH-associated lipoamide.[101] The components other than E1 in the PDH complex, in conjunction with the coenzymes FAD (flavin adenine dinucleotide) and NAD, are responsible for the ultimate formation of acetyl coenzyme A.

Acetolactate synthase (EC 4.1.3.18) catalyses the formation of acetolactate from two molecules of pyruvate. The HETDP carbanion is formed in the usual way, but instead of being protonated, it attacks the carbonyl carbon atom of a second molecule of pyruvate with the production of acetolactate (Figure 2.29).[104] Acetolactate synthase from plants, which is an enzyme in the branch-chain amino acid biosynthetic pathway, appears to go to great efforts to protect the carbanion or its equivalent from reaction with protons.[118] There is a flavin requirement in this enzyme, although the mechanism does not involve oxidation. It has been suggested that the flavin can protect the carbanion by an electron transfer mechanism should there be a lack of pyruvate in the organisms environment. Interference with the flavin causes destruction of enzyme activity. The powerful herbicide sulfometuron-methyl appears to function by interposing itself between the flavin and TDP.

**Figure 2.28**

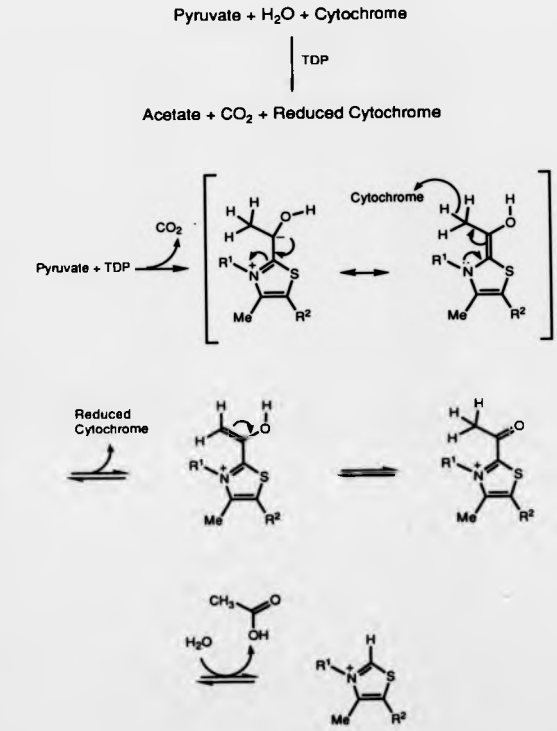
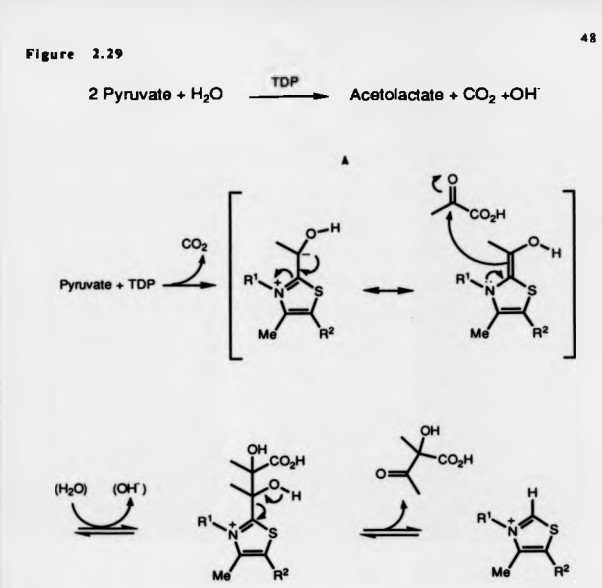

Pyruvate + $H_2O$ + Cytochrome
TDP
Acetate + $CO_2$ + Reduced Cytochrome
Pyruvate + TDP
$CO_2$
Cytochrome
Reduced Cytochrome
$H_2O$

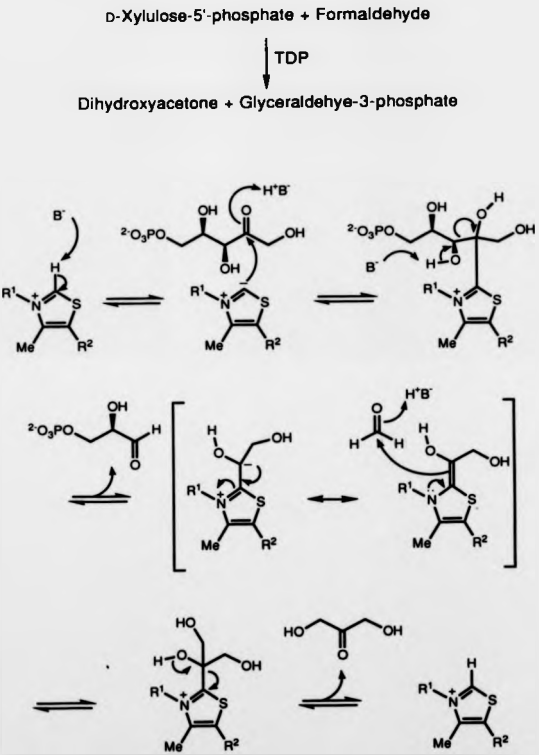
**Figure 2.29**




Transketolases perform reactions quite different from those above. For example, formaldehyde transketolase (EC 2.2.1.3) catalyses the conversion of D-xylulose-5'-phosphate and formaldehyde to dihydroxyacetone and glyceraldehyde-3-phosphate. The TDP ylide attacks the carbonyl carbon of D-xylulose-5'-phosphate with the subsequent release of glycerone to give the HETDP carbanion analogue, the carbanion of 2-(1,2-dihydroxyethyl)-TDP, which is also known as "active glycolaldehyde" (Figure 2.30). The 2-(1,2-dihydroxyethyl)-TDP carbanion is common to all transketolases and is not protonated in these enzymes. Instead, a new C-C bond is formed between the carbanion and an acceptor aldehyde. The resulting intermediate dissociates, in a manner analogous to that of HETDP, to form a new ketose phosphate.

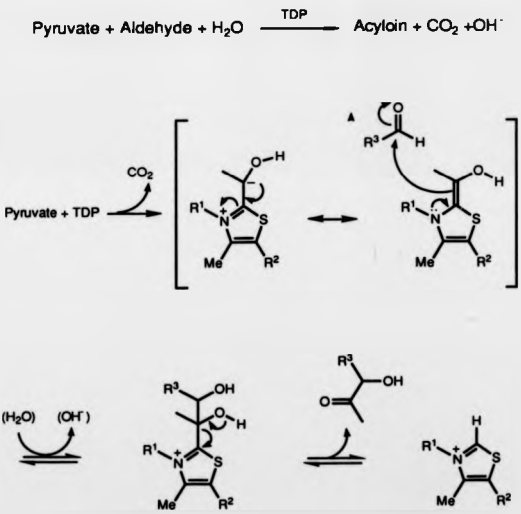
Transketolases therefore catalyse the transfer of active glycolaldehyde from one ketose to another.[119,120]

**Figure 2.30**



It has been proposed that PDC is in fact responsible for the formation of acyloins (see below). The mechanism is similar to that for acetolactate synthase. The HETDP carbanion is generated from pyruvate in the normal way, but instead of reacting with an 2-oxo acid, it attacks the carbonyl carbon of an acceptor aldehyde to from an new C-C bond (Figure 2.31). The final product is therefore an acyloin. Some biological roles for acetoin have been proposed (see above). However, the physiological role of general PDC-catalysed acyloin condensations is not understood.

**Figure 2.31**



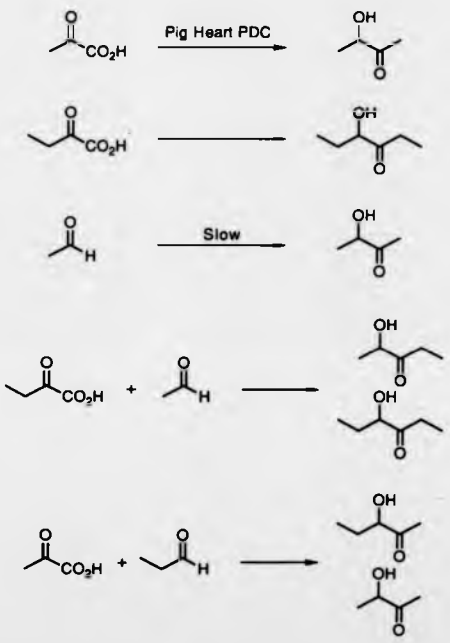
Advances in the understanding of the mechanism of TDP-dependent enzymes have been aided greatly by the study of non-enzymic thiamine catalysis. A chemical analogue of the PDC-catalysed acyloin condensation was first discovered by Mizuhara, who described the synthesis of acetoin from pyruvate and acetaldehyde using thiamine as a catalyst in alkaline conditions.[93] It has become apparent that thiamine does not catalyse some of the enzymatic reactions described above. For example, the decarboxylation of pyruvate leads to the formation of acetolactate (a 3-oxo acid that can thermally decarboxylate in acidic conditions to form acetoin) and not acetaldehyde.[121,122] Therefore, the architecture of the active site of each enzyme steers the course of the TDP dependent catalysis in a manner that is, as yet, not understood.[104]

**2.1.4.3** *Substrate specificity of PDC-catalysed acyloin formation.* The partially purified PDC from pig heart has been shown to catalyse the TDP and $Mg^{2+}$-dependent formation of acetoin from pyruvate with the evolution of carbon dioxide (Figure 2.32).[123] This reaction is practically quantitative, although acetoin is presumably not formed until some of the pyruvate has been converted to acetaldehyde. The analogous, but less rapid, conversion of 2-oxobutanoate to propionin (4-hydroxy-3-hexanone) was also demonstrated. The relative reaction rates reflect the decreasing affinity of 2-oxo acids with increasing chain length with regard to normal decarboxylation.[124-126] The corresponding acyloin products from either pyruvate or 2-oxobutanoate and either acetaldehyde or propanal were also described, illustrating some versatility in the reaction.[123]

Acetoin production was observed from acetaldehyde alone, although the reaction rate was very slow.[123] This can only be explained by the formation of the HETDP carbanion from acetaldehyde rather than pyruvate.

demonstrating the reversibility of the post-decarboxylation steps. Propanal alone did not give detectable amounts of propionin.

**Figure 2.32**



Singer and Pensky found that the PDC from wheat germ catalysed the formation of acetoin from either pyruvate and acetaldehyde or acetaldehyde alone.[127,128] The maximum rate of acetoin formation was approximately 1/65 the initial uninhibited rate of pyruvate decarboxylation with wheat germ PDC. The rate of acetoin formation in the presence of pyruvate was between 2- and 4-fold the rate obtained with acetaldehyde alone, depending on the relative concentrations of pyruvate, acetaldehyde and the enzyme. When both acetaldehyde and pyruvate are present, maximum velocity is reached at approximately 5 mmol $dm^{-3}$ pyruvate and 50 mmol $dm^{-3}$ acetaldehyde. Half-saturation is reached at 1.3 mmol $dm^{-3}$ pyruvate and 8.6 mmol $dm^{-3}$ acetaldehyde. With acetaldehyde alone as substrate, saturation is reached at a concentration of about 200 mmol $dm^{-3}$ and half-saturation at 20 mmol $dm^{-3}$. (These figures must be viewed with caution as they were derived from single point determinations rather than from initial rate determinations.)

When pyruvate is the sole substrate, the amount of acetoin synthesised is less than 5% of the amount formed in the presence of excess acetaldehyde. It was suggested that acetaldehyde strongly inhibits the decarboxylation of pyruvate. Thus insufficient acetaldehyde is produced to overcome the low affinity for acetaldehyde in the production of acetoin.

Evidence that PDC was the active enzyme and not some contaminating activity came from the observation that inactivation by ageing, acid, heat treatment or 4-chloromercuribenzoate treatment resulted in an identical decline of both types of enzymic activities. Furthermore, the ratio of the specific activities of the two reactions remained constant throughout the enzyme purification. Finally, the purified enzyme appeared to be homogeneous and both activities had identical pH optima (6.2), TDP ($K_m$ = 1.35 - 1.44 μmol $dm^{-3}$) and $Mg^{2+}$ requirements.

**2.1.4.4** *Stereochemical aspects of acetoin formation.* It is well established that certain hydrolytic enzymes can act on both enantiomers of its substrate, although often at very different rates.[129] The extraordinary observation that the wheat germ enzyme produced acetoin in a partially optically active form was the first report of a partially asymmetric synthetic enzyme.[127,128] Since Singer's report, there have been very few clearly substantiated additional examples of partially asymmetric synthetic enzymes.[129]

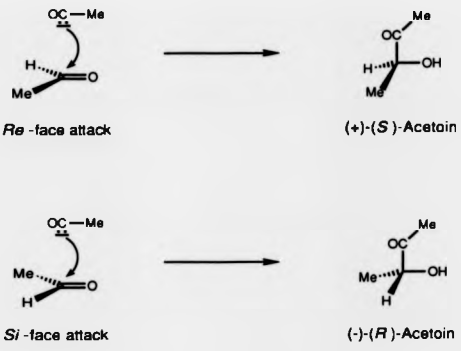
The acetoin produced from either pyruvate and acetaldehyde or acetaldehyde alone exhibited an optical rotation of $[\alpha]_D^{29} = 36 \pm 1°$.[127,128] The authors considered the most reliable specific optical rotation of enantiomerically pure acetoin to be -84 ± 2°, as extracted from filarial nematodes, *Aerobacter aerogenes* and various animal tissues.[3,130] This now appears to be correct as chemically synthesised (*R*)- and (*S*)-acetoin of high optical purity had specific rotations of -84 ± 3° and 82 ± 3°, respectively.[131] On the basis of the former figure and a rotation of 36°,[127,128] wheat germ PDC produced (*S*)-acetoin with an optical purity of 43% ee.

The presence of a racemase, the reversibility of the synthetic reaction and the occurrence of chemical racemisation on work up were ruled out as authentic (-)-(*R*)-acetoin, racemic acetoin and a preparation of predominantly (+)-(*S*)-acetoin were extracted quantitatively and without racemisation from control incubations with the enzyme.[127,128] If acetoin were produced non-enzymically with TDP, racemic acetoin would have been produced. This reaction does indeed occur at a pH of above 8.0.[101] However, the chemical reaction could not have taken place in the enzyme system as the solutions were buffered at pH 6.0. In addition, the optical purity of acetoin produced by wheat germ PDC was independent of the purity of the enzyme preparation. Interestingly, a number of crude plant meals had

previously been shown to produce acetoin from added pyruvate or acetaldehyde with an optical rotation of between 34° and 40°.[3]

Singer postulated that there may be two distinct active sites in wheat germ PDC which produce opposite enantiomers of acetoin at different rates.[127,128] However, he considered it more likely that there was in fact only one active site that was capable of forming acetoin. The partial asymmetric synthesis of acetoin could be rationalised as being as a result of the acceptor acetaldehyde molecule being attacked from two spacial directions by the active acetaldehyde (Figure 2.33). Therefore, the relative amounts of the two opposite enantiomers would represent the relative steric hindrances offered by the side-chains of the protein to the acceptor aldehyde, as it approaches the active acetaldehyde in the active site. Singer concluded that the uniform optical rotation of acetoin produced, presumably by PDC, in plants[3] indicated essentially the same active site in all plant PDCs, although they may differ sufficiently to be distinct immunological entities.

**Figure 2.33**




The PDC purified from yeast (YPDC) has also been shown to catalyse the formation of acetoin from pyruvate.[132,133] Small amounts of acetoin were also formed from acetaldehyde alone with this enzyme. Juni points out that this finding, coupled with the observation that acetolactate was not produced,[133] was evidence that the formation of acetoin did not occur *via* the decarboxylation of an acetolactate intermediate.

The maximum rate of acetoin formation was approximately 1/40 the initial uninhibited rate of pyruvate decarboxylation with YPDC. An acetaldehyde concentration of 20 mmol $dm^{-3}$ was determined to be saturating with respect to the formation of acetoin in the presence of pyruvate. The $K_m$ values for acetaldehyde and pyruvate were 5.2 and 1.5 mmol $dm^{-3}$, respectively, with regard to acetoin synthesis.

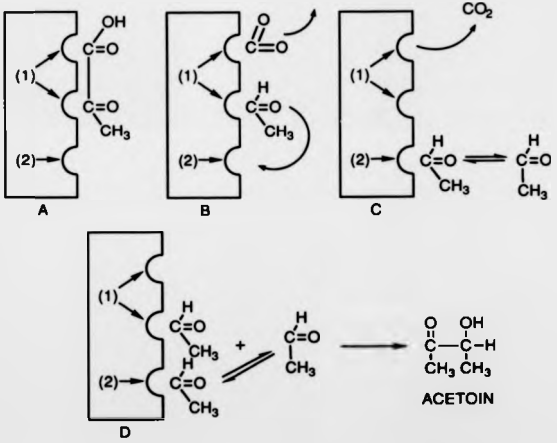
It has been known since the discovery of PDC in yeast by Neuberg that acetaldehyde inhibits the decarboxylation of pyruvate.[96] Juni showed that the rate of carbon dioxide evolution from pyruvate with YPDC decreased rapidly with time as acetaldehyde began to accumulate.[134] Other aldehydes, such as propanal, butanal, furfural and benzaldehyde, also inhibited the decarboxylation of pyruvate, although not as strongly as acetaldehyde. The inhibition of the reaction by these aldehydes did not appear to be competitive with respect to pyruvate. Further evidence that acetaldehyde is not a competitive inhibitor was provided by the $K_m$ value for pyruvate decarboxylation to acetaldehyde, which was an order of magnitude greater than that for the synthesis reaction. Acetaldehyde also inhibited the decarboxylation of other 2-oxo acids, such as 2-oxobutanoate and 2-oxopentanoate but the degree of inhibition decreased with increasing 2-oxo acid chain length.

If an excess of acetaldehyde was added initially, with pyruvate, the rate of carbon dioxide evolution was considerably inhibited but remained essentially constant with time since newly formed acetaldehyde did not alter appreciably the high acetaldehyde concentration. This finding also demonstrated that acetaldehyde inhibited the reaction but did not inactivate the enzyme. Although increasing initial acetaldehyde concentrations caused increasing inhibition of decarboxylation, the rate of the formation of acetoin remained essentially constant. At acetaldehyde concentrations exceeding approximately 0.25 mol $dm^{-3}$, the rate of decarboxylation approached the rate of acetoin synthesis.

Juni proposed a two-site mechanism to account for the action of YPDC as illustrated schematically in Figure 2.34.[134] Pyruvate binds to site 1 and is converted to active acetaldehyde. Free acetaldehyde is released only after the active acetaldehyde is transferred to site 2. If free acetaldehyde is present, it

will bind to site 2 and inhibit the decarboxylation of pyruvate and the production of acetaldehyde in a manner that is not competitive with respect to pyruvate. Acetaldehyde bound to site 2 will react with active acetaldehyde bound to site 1 to form acetoin. The observation that acetoin is not formed very rapidly from acetaldehyde alone can be explained by the virtual irreversibility of the transfer of active acetaldehyde from site 1 to site 2 in YPDC. By contrast, the PDC from pig heart and wheat germ are more efficient catalysts of this reaction,[123,127,128] presumably because the transfer of active acetaldehyde from site 1 to site 2 is essentially reversible in these enzymes.

**Figure 2.34**



Juni and Heym studied the effect of yeast proteases on YPDC.[135,136] YPDC was modified by one of these proteases in such a way as to diminish the acetaldehyde forming capacity and increase the acetoin forming activity. Thus, the modified YPDC lost its ability to direct pyruvate decarboxylation to form acetaldehyde and resembled the non-enzymic reactivity of thiamine, in that acetoin was the major product of pyruvate decarboxylation. Juni interpreted this result in terms of the specific modification of the second enzymic site.

Chen and Jordan estimated that the production of acetoin from pyruvate and acetaldehyde occurred some 80 ± 20 times faster than from acetaldehyde alone.[137] The $K_m$ and $V_{max}$ for acetaldehyde (alone) in the synthetic reaction was 1 mol $dm^{-3}$ and 140 μmol $dm^{-3}$ $min^{-1}$, respectively. By contrast, the $K_m$ (apparent) and $V_{max}$ for acetaldehyde, in the presence of 10 mmol $dm^{-3}$ pyruvate, in the synthetic reaction was 10.4 mmol $dm^{-3}$ and 90 μmol $dm^{-3}$ $min^{-1}$, respectively. The initial rate of formation of acetoin from pyruvate compared to that from acetaldehyde is expressed in the $K_m$s. Deuterium kinetic isotope effects suggest that C-H bond scission is part rate limiting in the formation of acetoin from acetaldehyde. This means that the formation of the HETDP carbanion from HETDP is part rate limiting.

The optical purity of (*R*)-acetoin produced by YPDC from pyruvate was 54% ee, as determined by optical rotation. Thus both the yeast and wheat germ[127,128] enzyme do not produce optically pure acetoin. However, the surprising result is that they produce predominantly opposite enantiomers of acetoin. This intriguing finding was compounded by the observation that (*R*)-acetoin produced with the yeast enzyme from acetaldehyde alone was of only 27% ee.[137] However, this change in optical purity must be treated with caution as the optical rotation measurements could have been affected by other, optically active chiral contaminants.

Crout *et al.* confirmed that the PDC from wheat germ produces predominantly (*S*)-acetoin from pyruvate and acetaldehyde, although the optical purity was determined by dilution analysis, against a crystalline derivative of optically pure acetoin, to be only 16% ee.[138] A surprising result was the formation of racemic acetoin from pyruvate when no acetaldehyde was added. It was suggested that a two-site mechanism, similar but not identical to that proposed by Juni (Figure 2.34),[134] may account for this result.

Little is known regarding the stereochemistry of PDC-catalysed decarboxylation reactions. There appears to be only one report of the decarboxylation of a chiral 2-oxo acid. (+)-3-Methyl-2-oxopentanoate was decarboxylated by YPDC at the same rate as pyruvate, but the (-)- isomer was decarboxylated at one quarter the rate.[139]

Lactyl-TDP, the HETDP carbanion and HETDP are a chiral molecules. A specific optical rotation of -10° ± 2 for HETDP isolated from pig heart PDH was reported.[109] (-)-HETDP is now known to have the (*S*)-absolute configuration, although the optical purity of the isolated HETDP is unknown.[140] Both of the chemically synthesised optical isomers of HETDP have been shown to be converted to TDP and acetaldehyde by the apoenzyme of wheat germ PDC.[140,141] The $K_m$ values for these two substrates were similar. It was suggested that the $K_m$ values depended on the rate of association of the apoenzyme with the coenzyme rather than the rate of the conversion process. Therefore it may be that the enzyme generates only one enantiomer in the normal decarboxylation reaction. The stereochemistry of lactyl-TDP and the HETDP carbanion are unknown. Although the stereochemistry of these intermediates is important, the chiral centre is lost on liberation of either the aldehyde or acyloin product.

**2.1.4.5** *Further acyloin products of PDC.* Shaw and Westerfeld found that beef heart PDC catalysed the formation of 4-hydroxy-5-oxohexanoic acid from pyruvate and succinic semialdehyde (3-formylpropanoic acid) (Figure 2.35).[142] This is apparently the only example of the enzymic formation of an acyloin compound from an aldehyde acceptor with a carboxylic acid function.

**Figure 2.35**


Although Neuberg had attributed PAC formation to the action of carboligase in 1921,[19] it was only in 1988 that purified YPDC was shown by Bringer-Meyer and Sahm to catalyse this reaction.[10] (See also.[23]) They also compared YPDC with the PDC from the obligately fermantative[143] bacterium *Zymomonas mobilis* (ZMPDC) with regard to acetoin and PAC formation. The $K_m$ value for acetaldehyde in the synthesis of acetoin by ZMPDC was found to be greater than that for YPDC, although the $V_{max}$ values for each enzyme were virtually identical. Similarly, the $K_m$ values for benzaldehyde in the synthesis of PAC by ZMPDC and by YPDC were estimated to be 125 and 50 mmol $dm^{-3}$, respectively. Saturating conditions with respect to benzaldehyde could not be established since the solubility of this substrate in aqueous solution was estimated to be approximately 90 - 100 mmol $dm^{-3}$. In addition, benzaldehyde was found to inhibit YPDC activity at a concentration exceeding 66 mmol $dm^{-3}$.[24]

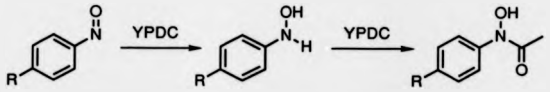
The YPDC was known to have a strongly hydrophobic active site in order for the enzyme to stabilise the HETDP carbanion over a sufficient lifetime for further conversion.[144,145] It was suggested that the relatively higher $K_m$ values for ZMPDC were as a result of the relatively less hydrophobic active site of this enzyme, as compared with YPDC.[10] This was confirmed by the fact that with ZMPDC, the $K_m$ for 2-oxo acid decarboxylation increased with increasing chain length. By contrast with YPDC, the $K_m$ did not change appreciably with increasing chain length.[125] Further evidence came from the three-fold higher $K_i$ of a fluorescent competitive inhibitor with ZMPDC compared with that obtained with YPDC.[10,146]

The fact that PDC contributes 4-6% of the soluble protein of *Z. mobilis* cells[147,148] and that these cells contain a five-fold higher PDC activity compared with *S. carlsbergensis* cells[148] prompted Bringer-Meyer and Sahm to compare the PAC forming activity of these two types of cells.[10] Under standard conditions, *S. carlsbergensis* cells formed over four times as much PAC as *Z. mobilis* cells. Since both microorganisms produced similar amounts of benzyl alcohol, it was concluded that the reason for this difference in PAC forming capacity was due to the unfavourable $K_m$ of ZMPDC for benzaldehyde. Another possible reason for this difference may have been the fact that intracellular pyruvate concentration, rather than PDC activity, is rate determining in the whole-cell biotransformation.[27,149]

A fascinating extension of PDC-catalysed acyloin condensations has been described by Corbett *et al.*[150] YPDC was found to catalyse the formation of *N*-phenylacetohydroxamic acid from nitrosobenzene and pyruvate (Figure 2.36). This is directly analogous to PAC formation where the carbonyl group of benzaldehyde was substituted by a nitroso group. Several 4-substituted nitrosobenzenes were also found to be suitable substrates.[151] Using 4-chloronitrosobenzene as a model substrate, it was established that the major

product was 4-chlorophenylhydroxylamine. Corbett and Chipko suggested that this reduction product was formed with the concomitant oxidation of the HETDP carbanion intermediate to acetyl-TDP, which was subsequently capable of acetylating the hydroxylamine to form the hydroxamic acid. This mechanism, quite distinct from the normal mechanism of acyloin condensations, seems to be restricted to this system since no benzyl alcohol is detected in the PDC-catalysed formation of PAC from benzaldehyde.

**Figure 2.36**




R = H, Cl, Br

The formation of *N*-(4-chlorophenyl)glycolhydroxamic acid from 4-chloronitrosobenzene and fructose-6-phosphate was also reported to be catalysed by yeast transketolase (Figure 2.37).[151] The lack of the the hydroxylamine product in this system suggested that the transketolase utilised 4-chloronitrosobenzene in the same manner as it would with its normal acceptor aldehyde cosubstrate, glyceraldehyde-3-phosphate.

**Figure 2.37**


There appear to be only two reports of the formation of an acyloin from a diacidic 2-oxo acid donor. Firstly, Shaw and Westerfeld described kinetic evidence for the enzymic formation of 5-hydroxy-4-oxohexanoic acid from 2-oxoglutarate and acetaldehyde (Figure 2.38).[142] Evidence was presented which indicated that the PDC from beef heart was the active enzyme.

**Figure 2.38**


Secondly, Davies and Kenworthy have reported the synergistic decarboxylation of glyoxylate (formylmethanoic acid) and 2-oxoglutarate by wheat germ PDC (Figure 2.39).[152] It was proposed that 2-oxoglutarate was decarboxylated to the corresponding TDP carbanion intermediate, which then condensed with glyoxylate to form 2-hydroxy-3-oxoadipic acid. 5-Hydroxylaevulinic acid was tentatively identified as the final product. This final product would result from the thermal decarboxylation of the 3-oxo acid 2-hydroxy-3-oxoadipic acid on acid work-up. Kinetic experiments suggest that acyloin compounds are also formed when glyoxylate is substituted by

pyruvate, formaldehyde (methanal), acetaldehyde, propanal, glycolaldehyde (hydroxyethanal) and glyceraldehyde (Figure 2.40). It appears that beef heart PDC is also capable of the formation of 2-hydroxy-3-oxoadipic acid from glyoxylate and 2-oxoglutarate.[142] By contrast, it seems that YPDC is not capable of forming acyloin compounds from 2-oxoglutarate since this enzyme does not decarboxylate this substrate unless it is in the form of the 5-ethyl ester.[70]

**Figure 2.39**

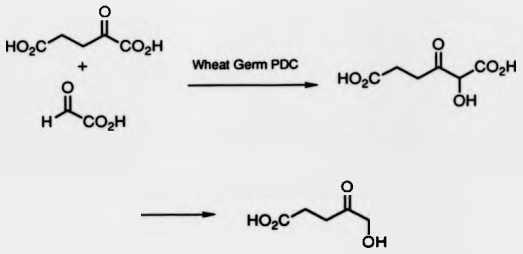
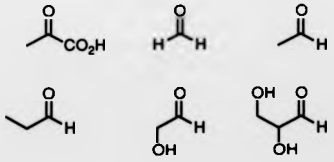
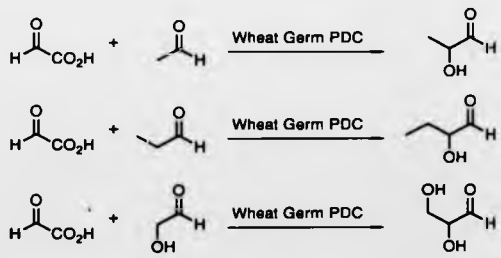



**Figure 2.40**



An interesting example of the formation of an acyloin compound from a 2-oxo acid donor other than pyruvate was described by Davies and Corbett.[153,154] Lactaldehyde (2-hydroxypropanal) was tentatively identified as the product formed from pyruvate and glyoxylate by purified wheat germ PDC (Figure 2.41). Glyoxylate was found to be decarboxylated by wheat germ PDC resulting in the inhibition of the enzyme with regard to pyruvate decarboxylation. However, pyruvate, acetaldehyde and glycolaldehyde stimulated pyruvate decarboxylation. The suggested explanation of this anomaly was that the rate determining step in glyoxylate decarboxylation was the removal of the product, formaldehyde, from the enzyme. Indeed, the degree of carbon dioxide evolution from glyoxylate always exceeded the formation of free formaldehyde. In other words, active formaldehyde was relatively stable with respect to protonation, but capable of forming the acyloin compound, lactaldehyde, with acetaldehyde. Similarly, 2-hydroxybutanal and glyceraldehyde were tentatively identified as the acyloin products formed from glyoxylate with propanal and glycolaldehyde, respectively (Figure 2.41).

**Figure 2.41**



The observation that glyoxylate is decarboxylated without the stoicheiometric release of formaldehyde was also made with YPDC.[155,156] Indeed glyoxylate was found to be an active centre label of YPDC since 2-hydroxymethyl-TDP was isolated from an incubation mixture of the enzyme and glyoxylate.[156]

**2.1.4.6** *Enzymes other than PDC involved in the metabolism of acyloins.* It must be pointed out that the acyloin acetoin can also be formed in biological systems by the decarboxylation of acetolactate.[138] Acetolactate decarboxylase from *A. aerogenes* (= *Klebsiella aerogenes*) catalyses the conversion of (*S*)-2-acetolactate to (-)-(*R*)-acetoin and is essentially inactive towards (*R*)-2-acetolactate. This enzyme seems to be the dominant acetoin forming activity in many organisms and tissues, such as filarial nematodes, *A. aerogenes* and various animal tissues because essentially optically pure (*R*)-acetoin was isolated from these tissues.[3,130] By contrast, purified wheat germ PDC[127,128,138] and various plant meals[3] produce (*S*)-acetoin with a low to moderate optical purity, suggesting that PDC is the predominant acetoin forming activity in these organisms.

It must be stated however, that optically active acetoin can be formed from either 2,3-butandiol or 2,3-butandione by microorganisms that possess the requisite stereospecific dehydrogenases (for example[1,9,157,158]), which are analogous to those discussed above in relation to PAC synthesis by yeast cells.

**2.1.4.7** *Kinetic and structural aspects of PDC.* ZMPDC exhibits normal saturation kinetics.[147,159] By contrast, YPDC[146,160,161], the PDC from sweet potato roots,[162,163] the PDC from *Zea mays* (maize)[164-167] and wheat germ PDC[168] do not obey normal saturation kinetics with respect to the decarboxylation of pyruvate. Pyruvate, itself, is the physiological allosteric activator of these enzymes. The product of pyruvate decarboxylation, acetaldehyde, is also a weak allosteric activator.[160] One of the most potent

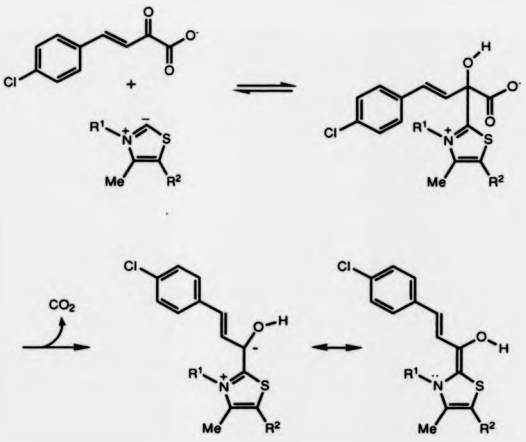
activators, pyruvamide, is almost as effective as pyruvate.[160] Pyruvamide does not inhibit enzymic pyruvate decarboxylation, and therefore has been used as an enzyme regulator which does not interfere with the catalytic site.

YPDC is essentially inactive in the absence of its substrate or any other activator.[169,170] In addition, inhibitors require the enzyme to be allostericaly activated in order to manifest their inhibitory effects.[171] Thus the process of allosteric activation is essential for the enzyme to function.

The activation process involves the reversible formation of a thiohemiacetal between the activator molecule and a cyteine residue in a regulatory site of the enzyme, which is distinct from the catalytic site.[116,171] The two-site mechanism of allosteric activation coined by Ullrich[146] must therefore not be confused with the two-site mechanism of acetoin synthesis proposed by Juni.[134] Binding of the activator molecule leads to the enzyme undergoing a conformational change such that it adopts a more "open" and flexible structure.[172-174] This conformational change results in the greater mobility of the active site.[175]

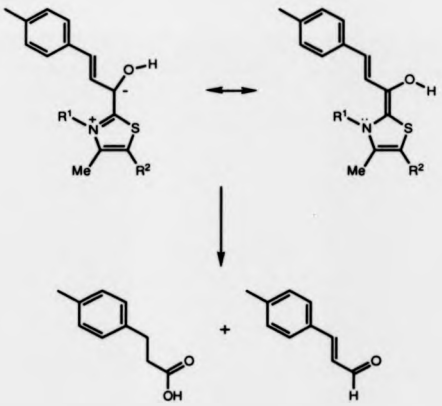
The conjugated 2-oxo acid, (*E*)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid was found to be suicide substrate for YPDC.[176-179] Spectroscopic evidence (UV-visible) was consistent with the formation of a stable enamine intermediate (Figure 2.42). Kinetic studies indicated that the formation of the enamine was dependent on the allosteric activation of the enzyme and that the suicide substrate was a relatively weak allosteric activator.

**Figure 2.42**



The allosteric activator pyruvamide was found to enhance the rate of enamine formation from (*E*)-4-(4-tolyl)-2-oxo-3-butenoic acid by as much as 50-fold.[180] The compound underwent catalytic turnover, leading to an enamine intermediate which could be protonated at both allylic positions leading to *p*-methylcinnamaldehyde and *p*-methyldihydrocinnamic acid in the ratio of 1:3 in the absence of and 3:2 in the presence of the allosteric activator pyruvamide (Figure 2.43). Thus pyruvamide can modulate the rate and stereochemistry of PDC-catalysed reactions.

**Figure 2.43**




PDC has also been purified from *Erwinia amylovora*,[181] germinating pea[182] and bean[183] seeds, orange,[184] bovine brain,[185,186] *Oryza sativa*[187] and guinea pig.[188] All of the PDCs isolated to date have many characteristics in common. They share similar tetrameric subunit structures, where each subunit binds one molecule of TDP and a magnesium ion in a single active site.

The genes coding for YPDC (*PDC1*)[189] and ZMPDC[190-194] have been cloned and sequenced. The amino acid sequences derived from the genes are homologous.[193,195] In addition, they share homology with the TDP-dependent enzymes acetolactate synthase, pyruvate oxidase (cytochrome)[195] and benzoylformate decarboxylase.[196] The E1 subunit of pyruvate dehydrogenase, rather surprisingly, is not homologous with the above

enzymes.[195] However, this enzyme does share a putative TDP binding sequence motif with the above enzymes and, amongst others, formaldehyde transketolase.[197]

Table 2.4 shows a comparison of some of the characteristics of YPDC and ZMPDC. It is clear that these enzymes are very similar in many respects. However, a notable exception is the fact that YPDC is allosterically activated, whereas ZMPDC is not.

**Table 2.4** A comparison of some of the characteristics of YPDC and ZMPDC

| Characteristic | YPDC | ZMPDC |
|---|---|---|
| Molecular weight/$10^{-3}$ | 230-240[a-c] | 200-240[d-f] |
| Subunit structure | $\alpha_4$[a,c,g-l] | $\alpha_4$[d,f] |
| | $\alpha_2\beta_2$[b,h,m-p] | |
| Subunit molecular weight/$10^{-3}$ | 59-65[a,b,k] ($\alpha_4$) | 57[d], 59[f] |
| | 55-59; 61-62[b,h,n] ($\alpha_2\beta_2$) | |
| Cofactors | $Mg^{2+}$, TDP[a] | $Mg^{2+}$, TDP[d] |
| Isoelectric point/pI | 5.8[l], 5.1-5.2[c] | 4.87[d] |
| Specific activity/U $mg^{-1}$ | 25-75[n,i,k], 80-85[o,q,r] | 181[d], 130-134[e,f] |
| Pyruvate affinity ($K_m$/mmol $dm^{-3}$) | 0.85-1.3[h,v,w], 2-8[s-u] | 0.3-0.4[d,f], 4.4[e] |
| TDP affinity ($K_m$/μmol $dm^{-3}$) | 1.35-1.44[x] | 1.28[d] |
| $Mg^{2+}$ affinity ($K_m$/μmol $dm^{-3}$) | N.f.[y] | 24[d] |
| pH Activity optimum | 5.8-6.2[k,z,α] | 6.0-6.5[e,f] |
| pH Stability optimum | 5.0-7.0[α] | 6.0- 7.0[f] |

[a]Lit.[198], [b]Lit.[199], [c]Lit.[200], [d]Lit.[148], [e]Lit.[159], [f]Lit.[147], [g]Lit.[201], [h]Lit.[202]. See below. [i]Lit.[203], [j]Lit.[204], [k]Lit.[205], [l]Lit.[206], [m]Lit.[207], [n]Lit.[208], [o]Lit.[209], [p]Lit.[210], [q]Lit.[211], [r]Lit.[212], [s]Lit.[125], [t]Lit.[213], [u]Lit.[146], [v]Lit.[155,214], [w]Lit.[161], [x]Lit.[127,128]. [y]Not found. [z]Lit.[137], [α]Lit.[144].

ZMPDC has an $\alpha_4$ subunit structure.[147,148] Therefore a further difference between ZMPDC and YPDC is the finding that YPDC can be isolated with either an $\alpha_2\beta_2$ subunit structure or an $\alpha_4$ subunit structure.[198,200,201,203,204,206] Evidence for two distinct subunits came from peptide mapping,[207] electrophoretic[202,208,209] and amino acid sequence analyses.[210] Kuo *et al* isolated two YPDC isozymes, one with an $\alpha_2\beta_2$ subunit structure and another with an $\alpha_4$ subunit structure, from a single yeast strain.[202] The YPDC isozymes have virtually identical properties. One may conclude that the type of YPDC subunit structure that is observed may depend on the yeast strain under investigation since there is compelling evidence for both types of subunit structure.

The genetics of PDC in yeast provides further information. A gene coding for PDC in *S. cerevisiae* has been cloned [204], sequenced [189] and named *PDC1*. Active PDC is over-produced when *PDC1* is cloned into a multi-copy plasmid [204] and *PDC1* is therefore a structural gene. Transcription of the *PDC1* gene is under metabolic control.[204,215,216]

The evidence for two distinct PDC subunits has encouraged many groups to search for further yeast *PDC* genes. Genes homologous to *PDC1* have been identified.[204,217,218] However, it was concluded that these genes were not structural genes but either pseudogenes or pyruvate dehydrogenase complex (E1 subunit) genes. A further *PDC* gene, *PDC2*, has been identified but it was concluded that it was not a structural gene.[204] *PDC2* has most probably a regulatory role. Similarly, *PDC3* [219] and *PDC4* [220] are also thought to have regulatory roles.

A second *PDC* structural gene, *PDC5*, has recently been identified.[213,221] *PDC1* and *PDC5* are 88% identical. *PDC5* is responsible for PDC activity in *PDC1* deletion mutants. The homo-tetramers expressed by these two genes have

similar affinities for pyruvate and identical affinities for TDP. *PDC5* is not expressed in *PDC1*$^+$ wild-type strains, presumably due feed-back inhibition, and therefore an $\alpha_2\beta_2$ subunit structure cannot be expected in this yeast strain. To conclude, two simultaneously expressed structural PDC genes have yet to be found in yeast.

**2.1.5** ***Aims.***— Significant advances in the understanding of whole-cell-catalysed formation of acyloins have been made. However, relatively little is known about the enzymology of acyloin condensations. In addition, there is a considerable lack of information about the stereochemistry of these reactions, particularly with respect to the optical purity of the products obtained.

The aim of this study was to investigate the substrate specificity and stereochemistry of ZMPDC-catalysed acyloin condensations and compare the results with those obtained with YPDC and PDCs from other sources. A greater understanding of the enzymology of acyloin formation would be of benefit in developing whole-cell biotransformations. Further more, the use of isolated enzymes may have practical advantages over whole-cell biotransformations.

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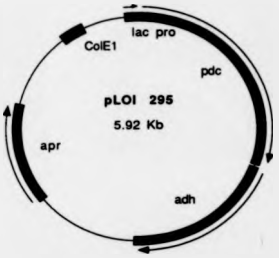
## 2.2 Results

**2.2.1** *Purification of ZMPDC.*— Brewer's yeast PDC (YPDC) is commercially available as a partially purified preparation. However, *Zymomonas mobilis* PDC (ZMPDC) is not commercially available. Ingram *et al.* cloned and sequenced the genes coding for ZMPDC[192] and *Z. mobilis* alcohol dehydrogenase II[222] from *Z. mobilis* strain CP4 ATCC 31821 and they incorporated these genes into the multi-copy plasmid pLOI295 (Figure 2.44).[223,224] Ingram *et al.* found that the *Z. mobilis* genes were strongly expressed in *Escherichia coli* under the control of the *lac* promoter.[223,224]

**Figure 2.44**




Ampicillin resistance gene (apr), the *Z. mobilis* strain CP4 ATCC 31821 genes for ZMPDC (pdc) and alcohol dehydrogenase II (adh) in the plasmid pLOI295 under the control of the *E. coli lac* promoter (lac pro).[223,224]

In the present study, the specific PDC activities of cell-free extracts of wild-type *E. coli* strain DH1, recombinant *E. coli* containing pLOI295 and wild-type *Z. mobilis* strain CP4 ATCC 31821 were determined (Table 2.5). Endogenous

NADH + $H^+$ oxidase and lactate dehydrogenase activities could have interfered with the assay.[192] However, the cell-free extracts were heat treated to inactivate these heat labile interfering activities, leaving the heat stable ZMPDC activity intact.

Table 2.5 Specific PDC activity of cell-free extracts[a]

| Strain | PDC Specific activity U $mg^{-1}$ |
|---|---|
| *E. coli* | 0.0 |
| *E. coli* + pLOI295 | 49.7 |
| *Z. mobilis* | 1.1 |

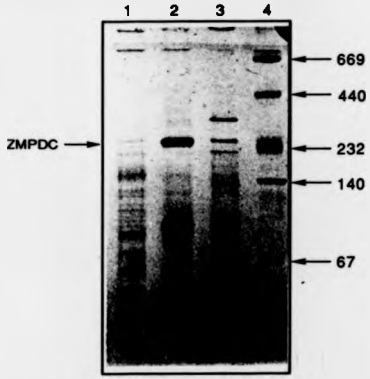
[a]Cells were harvested from 2 $dm^3$ fermentations. The cell-free extracts were heat treated (60 °C; 10 min) prior to the determination of specific PDC activity.

The wild-type *E. coli* strain was found to be a suitable host for pLOI295 because it did not have any detectable endogenous PDC activity. The recombinant *E. coli* strain exhibited a 50-fold higher PDC activity than the wild-type *Z. mobilis* strain confirming the extremely high level of expression of the cloned *Z. mobilis* genes under the control of the *lac* promoter in *E. coli*. Assuming that the specific activity of pure[148] ZMPDC is 181 U $mg^{-1}$, the recombinant ZMPDC comprised 27% of the soluble protein of the transformed *E. coli* cells.

Non-denaturing polyacrylamide electrophoresis (PAGE) of the cell-free extracts showed the presence of two strongly staining proteins in the

recombinant *E. coli* that were not present in the wild-type *E. coli* strain (Figure 2.45). The molecular weights of these proteins (approximately 255 000 and 100 000) are consistent with the expression of ZMPDC and *Z. mobilis* alcohol dehydrogenase II (lit., 200 000-240 000[147,148,159] and 147 300,[159] respectively) with the correct quaternary structures.

**Figure 2.45** Non-denaturing PAGE of cell-free extracts

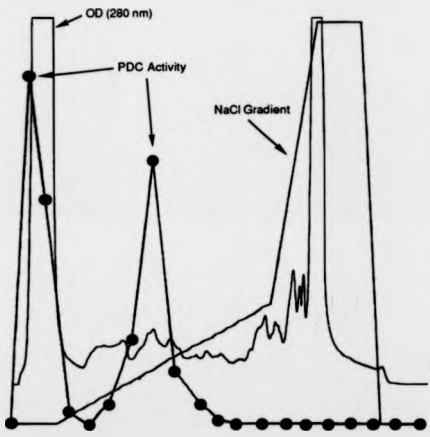



Electrophoretic separation of the soluble proteins of *E. coli* (channel 1), recombinant *E. coli* (2), *Z. mobilis* (3) and molecular weight markers (4). Molecular weights are shown in thousands. The gel was stained with Coomassie Blue.

It was concluded that the the best source of the enzyme was the recombinant *E. coli*. In order to be confident that the recombinant enzyme was expressed and folded correctly the recombinant enzyme required comparison with the wild-type enzyme. To that end, both enzymes were purified.

In an attempt to purify the wild-type ZMPDC, a cell-free extract of *Z. mobilis* was heat treated at 60 °C for 10 min. The heat treated material was then applied to the anion exchange resin Mono Q (a resin bearing quaternary amine groups). A proportion of the activity was eluted from the resin with salt gradient (Figure 2.46). However, a significant amount of the activity eluted in the void volume before the salt gradient commenced.

**Figure 2.46** Mono Q anion exchange chromatography of heat treated *Z. mobilis* soluble protein

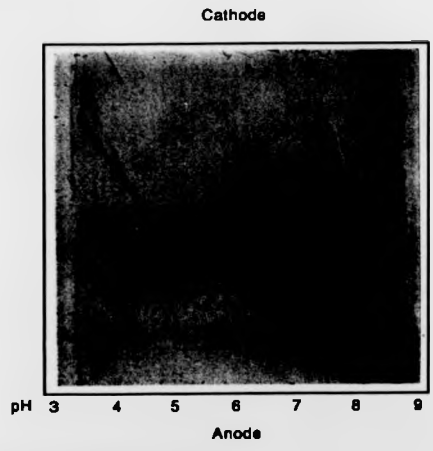



Heat treated *Z. mobilis* soluble protein (50 mg) was applied to a Mono Q anion exchange column and eluted with a 0 - 1 mol $dm^{-3}$ NaCl gradient in pH 7.0 bistrispropane buffer (20 mmol $dm^{-3}$). The vertical and horizontal scales are essentially arbitrary.

Since it has been reported[148] that ZMPDC has a pI of 4.87 and the column was equilibrated and eluted with pH 7.0 buffer, the active protein should have

bound to the column. The pI of ZMPDC was confirmed using 2-dimensional PAGE titration curve analysis of the cell-free extract of the recombinant *E. coli* (Figure 2.47). Assuming the most heavily stained protein is the recombinant ZMPDC (see Figure 2.45), the pI of the recombinant ZMPDC was estimated to be about 4.5. In addition, this protein was found to have a net negative charge at pH 7.0. Therefore, it seemed that some component(s) in the cell-free extracts were interfering with the binding of ZMPDC to the anion exchange resin.

**Figure 2.47** 2-Dimensional PAGE of the cell-free extract of the recombinant *E. coli*

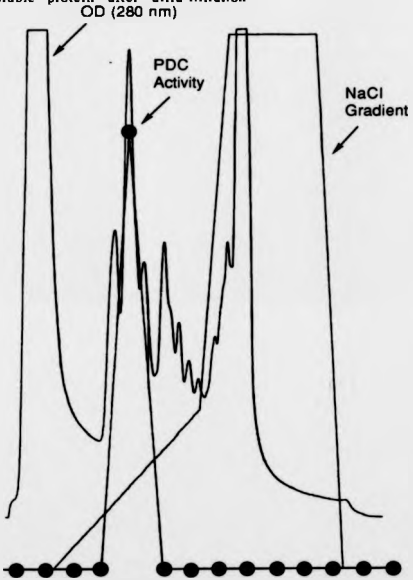



The gel was stained with Coomassie Blue.

In an attempt to remove the interfering components from the wild-type cell-free extract, material with a molecular weight below 10 000 was diluted 5-fold

by ultra-filtration. The resulting preparation was then heat treated and applied to the Mono Q column. This strategy was successful as the ZMPDC activity bound completely to the resin and was eluted with 0.11 mol $dm^{-3}$ NaCl (Figure 2.48).

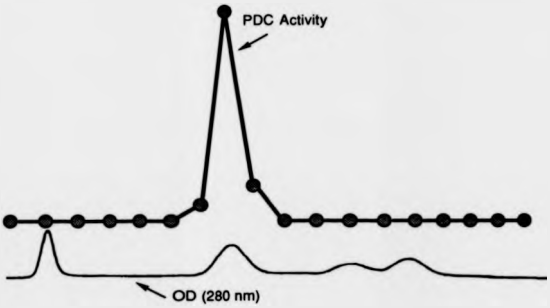
**Figure 2.48** Mono Q anion exchange chromatography of heat treated *Z. mobilis* soluble protein after ultra-filtration




Ultra-filtered and heat treated *Z. mobilis* soluble protein (50 mg) was applied to a Mono Q anion exchange column and eluted with a 0 - 1 mol $dm^{-3}$ NaCl gradient in pH 7.0 bistrispropane buffer (20 mmol $dm^{-3}$). The vertical and horizontal scales are essentially arbitrary.

Finally, the wild-type enzyme was applied to a Superdex 200 gel filtration column (Figure 2.49). The molecular weight of the wild-type enzyme was estimated to be 185 000 ± 7 000.

**Figure 2.49** Superdex 200 gel filtration of wild-type ZMPDC




Ultra-filtered and heat treated *Z. mobilis* soluble protein (5 mg) was applied to a Superdex 200 gel filtration column and eluted with pH 6.0 sodium citrate buffer (20 mmol $dm^{-3}$) containing NaCl (50 mmol $dm^{-3}$), TDP (1 mmol $dm^{-3}$), and $MgCl_2$ (10 mmol $dm^{-3}$). The vertical and horizontal scales are essentially arbitrary.

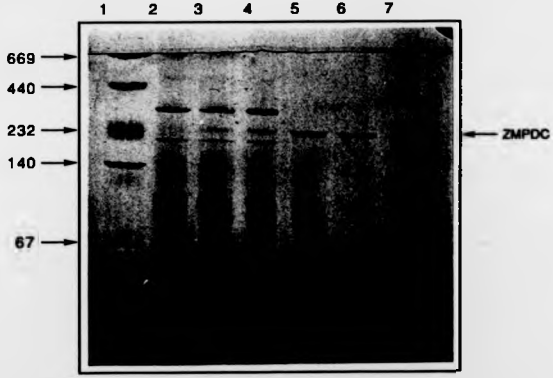
This procedure afforded a 24.3-fold purification and gave the wild-type enzyme with a specific activity of 97 U $mg^{-1}$ in 7% yield (Table 2.6 and Figure 2.50). The purity of the wild-type ZMPDC was sufficient for the comparison of this enzyme with the recombinant enzyme. The molecular weight of the wild-type enzyme was estimated using non-denaturing PAGE (Figure 2.50) to be 250 000 ± 4 000.

**Table 2.6** The purification of wild-type ZMPDC

| Step | Specific activity U mg$^{-1}$ | Yield U (%) | Fold-purification |
|---|---|---|---|
| Cell-free extract[a] | 4 | 700 (100) | 1.0 |
| Ultra-filtration | 5 | 451 (64) | 1.3 |
| Heat treatment | 11 | 381 (54) | 2.8 |
| Mono Q | 35 | 165 (24) | 8.8 |
| Superdex 200 | 97 | 48 (7) | 24.3 |

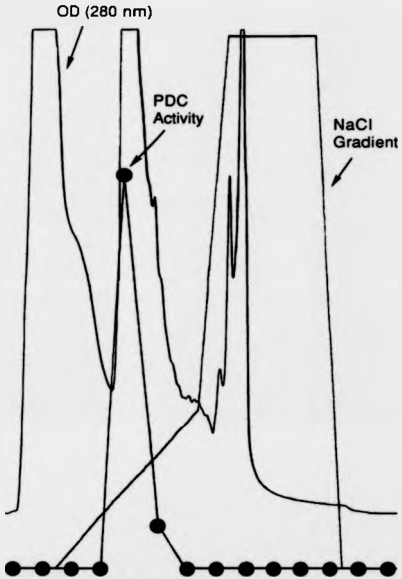
[a]A portion of cells were harvested from 20 dm$^{-3}$ fermentations.

**Figure 2.50** Non-denaturing PAGE of the purified wild-type ZMPDC




Electrophoretic separation of molecular weight markers (channel 1), *Z. mobilis* soluble protein (2), post-ultra-filtration (3), post-heat treatment (4), post-Mono Q (5), post-Superdex 200 (6) and molecular weight markers (7). Molecular weights are shown in thousands. The gel was stained with Coomassie Blue.

**Figure 2.51** Mono Q anion exchange chromatography of recombinant *E. coli* soluble protein




Ultra-filtered and heat treated recombinant *E. coli* soluble protein (50 mg) was applied to a Mono Q anion exchange column and eluted with a 0 - 1 mol $dm^{-3}$ NaCl gradient in pH 7.0 bistrispropane buffer (20 mmol $dm^{-3}$). The vertical and horizontal scales are essentially arbitrary.

The same procedure was used for the purification of the recombinant enzyme from the recombinant *E. coli*. It was found that the recombinant cell-free extract also required the ultra-filtration treatment in order for the active

protein to bind to the Mono Q resin efficiently (Figure 2.51). The activity eluted with 0.11 mol $dm^{-3}$ NaCl. The gel filtration step gave similar results to that shown in Figure 2.49. The molecular weight of the recombinant enzyme was estimated to be 190 000 ±7 000.

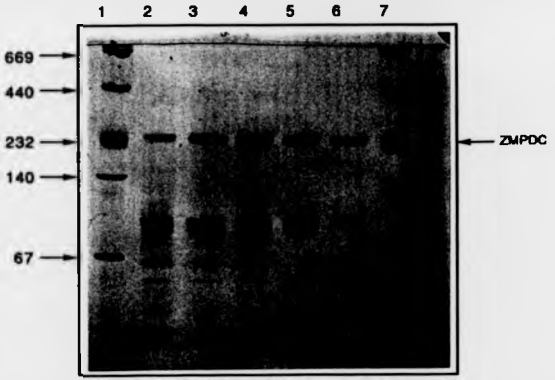
The recombinant ZMPDC was obtained with a specific activity of 150 U $mg^{-1}$ in 14% yield (Table 2.7 and Figure 2.52). The fold-purification was 5 times lower than that for the wild-type enzyme. However, the recombinant enzyme was obtained in with a higher specific activity because the specific activity of the recombinant cell-free extract was almost 8-fold higher than that for the wild-type. The molecular weight of the recombinant enzyme was estimated using non-denaturing PAGE (Figure 2.52) to be 246 000 ±4 000. The half life of the recombinant enzyme was estimated to be over 25 days when stored at 4 °C in the presence of the microbial inhibitor ethyl *p*-hydroxybenzoate. Long term storage of ZMPDC was possible at -20 °C in the presence of glycerol (50%, v/v) without a significant loss of activity over a period of months. The presence of glycerol did not affect the decarboxylation activity of ZMPDC.

**Table 2.7** The purification of recombinant ZMPDC

| Step | Specific activity U $mg^{-1}$ | Yield U (%) | Fold-purification |
|---|---|---|---|
| Cell-free extract[a] | 31 | 6 000 (100) | 1.0 |
| Ultra-filtration | 38 | 5 895 (98) | 1.2 |
| Heat treatment | 68 | 6 000 (100) | 2.2 |
| Mono Q | 115 | 3 700 (62) | 3.7 |
| Superdex 200 | 150 | 862 (14) | 4.8 |

[a] A portion of cells were harvested from 20 $dm^{-3}$ fermentations.

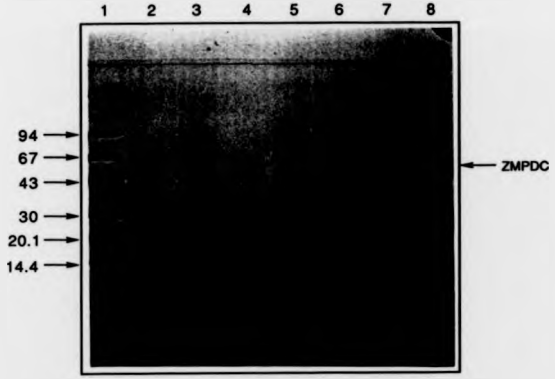
**Figure 2.52** Non-denaturing PAGE of the purified recombinant ZMPDC




Electrophoretic separation of molecular weight markers (channel 1), recombinant *E. coli* soluble protein (2), post-ultra-filtration (3), post-heat treatment (4), post-Mono Q (5), post-Superdex 200 (6) and molecular weight markers (7). Molecular weights are shown in thousands. The gel was stained with Coomassie Blue.

The molecular weights of the subunits of wild-type and recombinant ZMPDC were estimated using denaturing PAGE to be 65 000 ±4 000 (Figure 2.53). By comparison, YPDC obtained from Sigma contained a major protein of a similar molecular weight. However, this partially purified commercial preparation contained a number of additional proteins.

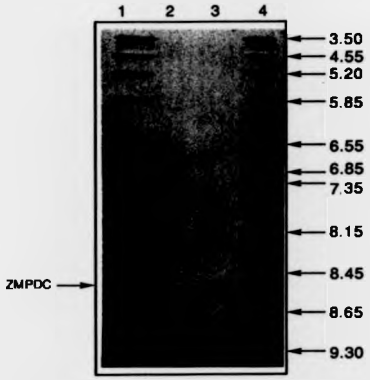
**Figure 2.53** Denaturing PAGE of purified wild-type and recombinant ZMPDC




Electrophoretic separation of molecular weight markers (channel 1), YPDC (Sigma; 2), *Z. mobilis* soluble protein (3), purified wild-type ZMPDC (4), molecular weight markers (5), purified recombinant ZMPDC (6), recombinant *E. coli* soluble protein (7) and *E. coli* soluble protein (8). Molecular weights are shown in thousands. The gel was stained with Coomassie Blue.

The pI of both *Z. mobilis* enzymes were estimated from isoelectric focusing PAGE to be 5.00 (Figure 2.54). The purified enzymes were also analysed by 2-dimensional PAGE. They gave similar results to those obtained with the recombinant cell-free extract (Figure 2.47), confirming the above interpretation.

**Figure 2.54** Isoelectric focusing PAGE of purified wild-type and recombinant ZMPDC




Isoelectric focusing of markers (channel 1), purified wild-type ZMPDC (2), purified recombinant ZMPDC (3) and markers (4). The gel was stained with Coomassie Blue.

It was assumed that the protein that was stained with Coomassie Blue in the purified enzyme preparations was ZMPDC. To check the identity of the stained proteins, a duplicate non-denaturing gel was incubated in the presence of pyruvate and then stained with 1,2-dianilinoethane, which forms a white insoluble compound with acetaldehyde.[225] It was found that ZMPDC was indeed the protein that was stained with Coomassie Blue. In addition, ZMPDC was detected in the recombinant *E. coli* cell-free extract. Furthermore, ZMPDC was the only PDC activity detected in this extract. ZMPDC was not detected in the *Z. mobilis* cell-free extract, presumably because the number of units of ZMPDC that were applied to the gel were insufficient.

**Figure 2.55** PDC activity stain of non-denaturing PAGE


Non-denaturing PAGE of recombinant *E. coli* cell-free extract (channel 1; 31 mU), purified recombinant ZMPDC (2; 57 mU), purified wild-type ZMPDC (3; 34 mU) and *Z. mobilis* cell-free extract (4; 4 mU). The gel was incubated in the presence of pyruvate and then stained with 1,2-dianilinoethane, which forms a white precipitate with acetaldehyde.

Acyloin compounds can be visualised with tetrazolium blue. The feasibility of a stain for acyloin compounds in polyacrylamide gels was assessed. The tetrazolium blue reagent contains methanol (50%, v/v) and NaOH (3 mol $dm^{-3}$) and is not a suitable reagent for use with polyacrylamide gels. Therefore, it was attempted to detect the presence of acyloin condensing activities on nitrocellulose diffusion blots of non-denaturing polyacrylamide gels. To reduce the detrimental effect of NaOH on the nitrocellulose, the NaOH concentration was reduced to 0.5 mol $dm^{-3}$. The nitrocellulose diffusion blots were placed on filter papers saturated with pH 6.0 sodium citrate buffer (50 mmol $dm^{-3}$) containing sodium pyruvate (0.5 mol $dm^{-3}$) and incubated at

ambient temperature for 1.5 h. No acetoin formation was detected. It was concluded that the efficiency of the diffusion blot and the detection limit (50 nmol of acetoin) were too low. It may be that a Western blot of a large slab gel would be more appropriate.

In summary, the recombinant ZMPDC appeared to be identical to the wild-type enzyme and the purified recombinant enzyme could be prepared in larger quantities. Therefore, the recombinant enzyme was used to study the formation of acyloin compounds.

**2.2.2** *Fractionation of YPDC.*— YPDC was commercially available in a partially purified form. The presence of contaminating proteins was confirmed by denaturing PAGE (Figure 2.53). To obtain a purer YPDC preparation, the commercial material was fractionated by Superdex 200 gel filtration chromatography.[#] The specific activity of YPDC was increased from 12 to 23 U $mg^{-1}$. It has been reported that the pure YPDC has a specific activity of 80—85 U $mg^{-1}$.[209,211,212] However, the fractionated YPDC appeared to be sufficiently purified on examination with denaturing PAGE for use in acyloin condensation reactions (Figure 2.56). The subunit molecular weight of YPDC appeared to be identical to that for ZMPDC and the presence of only one subunit type indicated that this YPDC preparation exhibited an $\alpha_4$ subunit structure.

The fractionated YPDC preparation was only used for the production of acyloin compounds where indicated.

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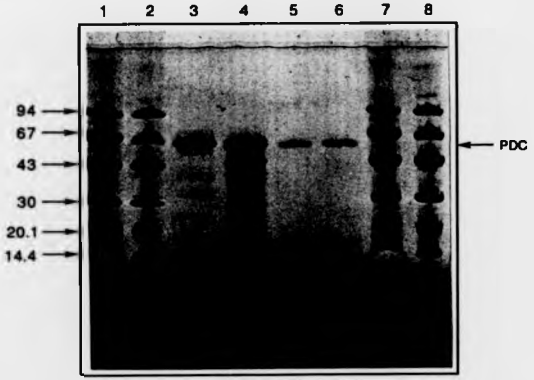
[#] The fractionation of YPDC was performed by Gregory Dean and Nick Thomson.

**Figure 2.56** Denaturing PAGE of fractionated YPDC



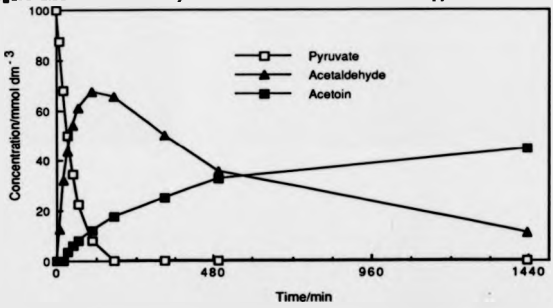

Electrophoretic separation of molecular weight markers (channels 1 and 2), purified recombinant ZMPDC (3), YPDC (Sigma; 4), fractionated YPDC (5 and 6) and molecular weight markers (7 and 8). Molecular weights are shown in thousands. The gel was stained with Coomassie Blue.

**2.2.3** ***The ZMPDC- and YPDC-catalysed formation of acetoin.***— ZMPDC catalysed the formation of acetoin (3-hydroxy-2-butanone) from pyruvate (Figure 2.57). The concentrations of pyruvate and acetoin were monitored by $^1$H NMR spectroscopy. During the initial part of the reaction, pyruvate was rapidly decarboxylated to acetaldehyde (Figure 2.58). In addition, acetoin was formed from pyruvate and acetaldehyde in the early stages of the reaction. The acetaldehyde concentration reached a maximum and then decreased as it is was consumed in the condensation to acetoin. The production of acetoin did not appear to decline when the pyruvate was exhausted, indicating that the

formation of acetoin from acetaldehyde alone was catalysed by ZMPDC at a rate comparable with that obtained with pyruvate and acetaldehyde. This finding was confirmed when ZMPDC was incubated with acetaldehyde alone (Figures 2.59 and 2.60). When both pyruvate and acetaldehyde were present initially, results were obtained which were consistent with those described above.

**Figure 2.57**

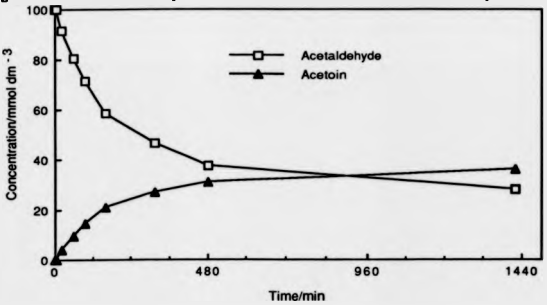

**Figure 2.58** ZMPDC-catalysed formation of acetoin from pyruvate




The reaction mixture contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 µmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), sodium pyruvate (100 mmol $dm^{-3}$) and ZMPDC (7.55 U) and was incubated at 30 °C. The substrates and products were monitored by $^1H$ NMR spectroscopy.

**Figure 2.59**

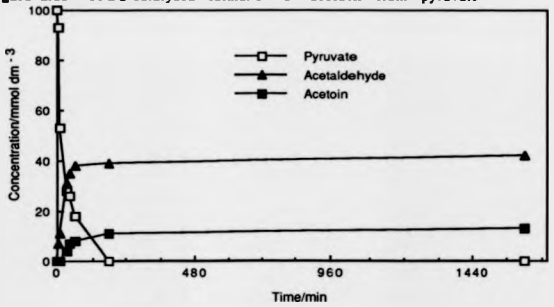
**Figure 2.60** ZMPDC-catalysed formation of acetoin from acetaldehyde alone




The reaction mixture contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 $\mu$mol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), acetaldehyde (100 mmol $dm^{-3}$) and ZMPDC (7.55 U) and was incubated at 30 °C. The substrates and products were monitored by $^1$H NMR spectroscopy.

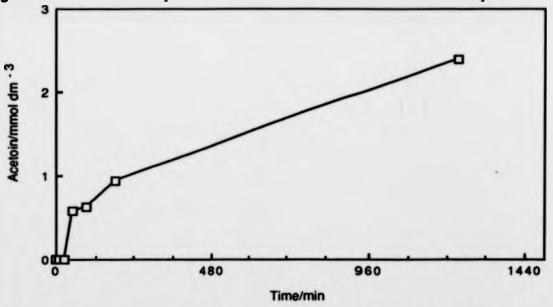
When YPDC was used instead of ZMPDC, quite different results were obtained. With pyruvate alone, YPDC catalysed the rapid formation of acetaldehyde with the concomitant and less rapid formation of acetoin (Figure 2.61). However, when the pyruvate was exhausted, the formation of acetoin virtually ceased. The formation of small amounts of additional unidentified products was observed after prolonged incubation. It has been suggested that activities other than PDC in the commercial YPDC preparation are responsible for these side reactions.[226] When YPDC was incubated with acetaldehyde alone, acetoin formation was not detected by 220 MHz $^1$H NMR spectroscopy. However, a rather low rate of acetoin formation was detected by GC (Figure 2.62).

**Figure 2.61** YPDC-catalysed formation of acetoin from pyruvate




The reaction mixture contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), sodium pyruvate (100 mmol $dm^{-3}$) and YPDC (7.55 U) and was incubated at 30 °C. The substrates and products were monitored by $^1H$ NMR spectroscopy.

**Figure 2.62** YPDC-catalysed formation of acetoin from acetaldehyde




The reaction mixture contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), acetaldehyde (100 mmol $dm^{-3}$) and YPDC (7.55 U) and was incubated at 30 °C. Acetoin production was monitored by GC.

The initial rates of pyruvate decarboxylation and acetoin formation were estimated from the studies described above for both ZMPDC and YPDC (Table 2.8). It is clear from these results that ZMPDC catalysed the formation of acetoin from acetaldehyde alone at a similar rate to that obtained from either pyruvate and acetaldehyde or pyruvate alone. By contrast, YPDC catalysed the formation of acetoin from acetaldehyde alone at a rate four orders of magnitude lower that that observed with ZMPDC. The YPDC-catalysed formation of acetoin from pyruvate alone was at a lower rate than that from pyruvate and acetaldehyde. This suggests that YPDC was not saturated with respect to acetoin formation in the former reaction.

**Table 2.8** The relative initial rates of pyruvate decarboxylation and acetoin formation catalysed by ZMPDC and YPDC[a]

| Reaction | Relative Rate of Reaction (%) | |
|---|---|---|
| | ZMPDC | YPDC |
| Pyruvate (100 mmol $dm^{-3}$) → Acetaldehyde | 100[b] | 100[b] |
| Pyruvate (100 mmol $dm^{-3}$) → Acetoin | 15[b] | 0.02[b] |
| Pyruvate (50 mmol $dm^{-3}$) + Acetaldehyde (50 mmol $dm^{-3}$) → Acetoin | 15[b] | 0.05[c] |
| Acetaldehyde (100 mmol $dm^{-3}$) → Acetoin | 14[b] | 0.001[c] |

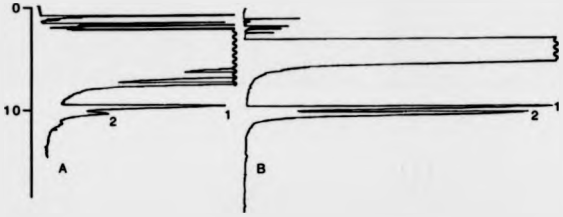
[a]Reaction mixtures contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$) and PDC (7.55 U) and were incubated at 30 °C.

[b]Determined by 220 MHz $^1H$ NMR spectroscopy.

[c]Determined by GC.

The optical purity of the acetoin produced by YPDC was determined by chiral GC (Figure 2.63). It was assumed that the major enantiomer produced by this enzyme was (*R*)-acetoin as reported previously.[137] The ee of acetoin appeared to decrease slightly with increasing incubation time (Table 2.9). Such a small degree of racemisation could not account for the fact that the acetoin was not optically pure. The racemisation of acetoin was not observed during the processes of solvent extraction and chiral analysis. In addition, the acetoin did not racemise when the extracts were stored at 4 °C over a period of two weeks. Furthermore, control reactions with either pyruvate or acetaldehyde and without enzyme did not produce acetoin. Thus, YPDC was responsible for the partial asymmetric synthesis of acetoin.

**Figure 2.63** The chiral analysis of acetoin produced by YPDC




The reaction mixture contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), sodium pyruvate (100 mmol $dm^{-3}$) and YPDC (7.55 U) and was incubated at 30 °C for 21 h. (*R*)-Acetoin (peak 1) and (*S*)-acetoin (peak 2) were resolved by chiral GC using a Lipodex A column at 20 °C (chromatogram A). The sample was co-injected with authentic racemic acetoin (B). Retention time is shown in min.

**Table 2.9** The optical purity of (*R*)-acetoin produced by YPDC[a]

| Substrate(s) (concentration/ mmol $dm^{-3}$) | Enantiomeric excess (%)[b] | |
|---|---|---|
| | 4 h | 21 h |
| Pyruvate (100) | 55, 51, 54 (53 ±2) | 45, 47, 45 (46 ±1) |
| Acetaldehyde (100) | N.d.[c] | 41, 44, 48 (44 ±4) |
| Pyruvate (100) and acetaldehyde (300) | 49, 49, 53 (50 ±2) | 45, 47, 46 (46 ±1) |
| Acetaldehyde (100) and pyruvamide (100) | 38, 37, 49 (41 ±7) | 42, 38, 38 (39 ±2) |
| Pyruvate (100) and glyoxylate (100) | N.d.[c] | 44, 35, 25 (35 ±10) |

[a]Triplicate reaction mixtures contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$) and YPDC (7.55 U) and were incubated at 30 °C.

[b]Determined by chiral GC. Aliquots were taken after 4 and 21 h incubation.

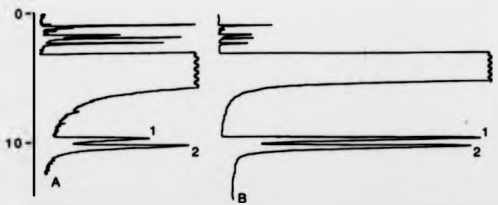
[c]Acetoin not detected.

There was no statistically significant variation in the ee of acetoin produced from pyruvate and/or acetaldehyde. The ee of acetoin appeared to be slightly lower when formed from acetaldehyde in the presence of the allosteric activator pyruvamide. However, this result must be treated with caution because this effect is very small. The presence of the inhibitor glyoxylate also appeared to reduce the ee of acetoin produced from pyruvate, although this result is not considered to be reliable since the amount of acetoin that was produced was barely above the limits of detection, resulting in the poor reproducibility of the chiral analysis.

An interesting observation was the greater conversion of acetaldehyde to acetoin in the presence of pyruvamide relative to that obtained with acetaldehyde alone. It was estimated that after 21 h a 4-fold higher conversion had occurred.

The corresponding data obtained with ZMPDC are shown in Figure 2.64 and Table 2.10. ZMPDC, like YPDC, did not produce optically pure acetoin. However, it was clear that ZMPDC produced predominantly the opposite enantiomer of acetoin that was formed with YPDC. There was no indication of any racemisation of acetoin during the incubation period. The optical purity of the acetoin seemed to be independent of the substrate and the presence of pyruvamide and glyoxylate. Pyruvamide had no effect on the rate of acetaldehyde condensation with this enzyme. Glyoxylate inhibited both enzymes to a similar extent.

**Figure 2.64** The chiral analysis of acetoin produced by ZMPDC




The reaction mixture contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 $\mu$mol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), sodium pyruvate (100 mmol $dm^{-3}$) and ZMPDC (7.55 U; 186 U $mg^{-1}$) and was incubated at 30 °C for 21 h. (*R*)-Acetoin (peak 1) and (*S*)-acetoin (peak 2) were resolved by chiral GC using a Lipodex A column at 20 °C (chromatogram A). The sample was co-injected with authentic racemic acetoin (B). Retention time is shown in min.

**Table 2.10** The optical purity of (*S*)-acetoin produced by ZMPDC[a]

| Substrate(s) (concentration/ mmol $dm^{-3}$) | Enantiomeric excess (%)[b] | |
|---|---|---|
| | 4 h | 21 h |
| Pyruvate (100) | 28, 28, 31 (29 ±2) | 31, 25, 29 (28 ±3) |
| Acetaldehyde (100) | 24, 26, 26 (25 ±1) | 29, 23, 24 (25 ±3) |
| Pyruvate (100) and acetaldehyde (300) | 30, 30, 28 (29 ±1) | 28, 28, 28 (28 ±0) |
| Acetaldehyde (100) and pyruvamide (100) | 28, 27, 23 (26 ±3) | 26, 22, 26 (25 ±2) |
| Pyruvate (100) and glyoxylate (100) | N.d.[c] | 29, 17, 26 (24 ±6) |

[a]Triplicate reaction mixtures contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$) and ZMPDC (7.55 U; 186 U $mg^{-1}$) and were incubated at 30 °C.

[b]Determined by chiral GC. Aliquots were taken after 4 and 21 h incubation.

[c]Acetoin not detected.

**2.2.4** *The ZMPDC- and YPDC-catalysed formation of lactaldehyde.*— The enzyme from both sources catalysed the formation of lactaldehyde (hydroxypropanal) from glyoxylate and either pyruvate or acetaldehyde (Figure 2.65 and Table 2.11). $^1H$ NMR spectra of the reaction mixtures spiked with authentic lactaldehyde confirmed the identity of this product. There was no indication of the production of either acetol (1-hydroxy-2-propanone) or any additional products other than lactaldehyde. No products were formed when glyoxylate was incubated in the presence of propanal.

**Figure 2.65**

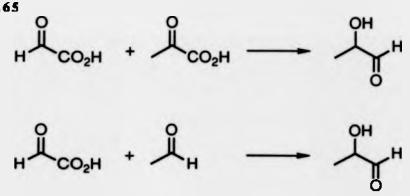


**Table 2.11** The PDC-catalysed production of lactaldehyde from glyoxylate[a]

| Co-substrate (concentration/ mmol $dm^{-3}$) | Lactaldehyde concentration[b]/mmol $dm^{-3}$ | |
|---|---|---|
| | ZMPDC | YPDC |
| Pyruvate (50) | 5 | 4 |
| Pyruvate (100) | 13 | N.d.[c] |
| Acetaldehyde (100) | 14 | 9 |

[a]Reaction mixtures contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), glyoxylate (100 mmol $dm^{-3}$) and either ZMPDC (7.55 U) or YPDC (7.30 U), and were incubated at 30 °C for 19 h.

[b]Determined by 220 MHz $^1H$ NMR.

[c]Not determined.

Glyoxylate severely, but not completely, inhibited the decarboxylation of pyruvate and the formation of acetoin. The order of addition of the substrates

and enzyme had no effect on the rate of reaction. These results indicated that glyoxylate was not an irreversible inhibitor.

Lactaldehyde was not detected in control reactions without either enzyme, acetaldehyde or pyruvate. Therefore, PDC was responsible for the synthesis of lactaldehyde where, presumably, glyoxylate is enzymically decarboxylated to form active formaldehyde which condenses with acetaldehyde to form the product. If free formaldehyde was formed, it appeared not to condense with active formaldehyde to give glycolaldehyde (2-hydroxyethanal). Lactaldehyde was not formed when the enzyme was incubated with acetoin and glyoxylate. Interestingly, this provided evidence that the enzymic formation of acetoin was irreversible.

Owing to the fact that lactaldehyde was a difficult compound to isolate from reaction mixtures, it was attempted to isolate the 2,4-dinitrophenylhydrazone derivative. This task proved to be very time consuming because the derivatised reaction mixtures required fractionation by both silica gel chromatography and HPLC. However, this method was successful and the lactaldehyde derivative was isolated in a pure form. The structure of this compound was unambiguously identified by comparison ($^1$H NMR spectroscopy, mass spectrometry, HPLC and TLC) with fully characterised authentic synthetic material.

It was of interest to determine the optical purity of lactaldehyde. The analysis of lactaldehyde by chiral GC was unsuitable because it is highly water soluble, and therefore difficult to extract, and it would be unlikely that lactaldehyde would exhibit a reasonable retention time using the Lipodex A column. $^1$H NMR spectroscopy (400 MHz; $CDCl_3$) of the 2,4-dinitrophenylhydrazone of synthetic racemic lactaldehyde in the presence of 10 molar equivalents of (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl)-ethanol

showed base-line splitting of the resonance of the aromatic proton at the 6 position. Although this method of chiral analysis was of practical value, it was not suitable for routine analysis.

A more appropriate HPLC method was developed. The authentic racemate was well resolved using a Chiralcel OB chiral column. The derivative of (*R*)-lactaldehyde, with the centre of chirality being derived from L-threonine (Figure 2.66), allowed the assignment of the HPLC peaks (Figure 2.67). Identical results were obtained when the eluted compounds were monitored at 350 nm. A co-injection with the racemate suggested that the minor impurity in the optically pure material was unlikely to be the other enantiomer.

**Figure 2.66** The synthesis of the 2,4-dinitrophenylhydrazone of (*R*)-lactaldehyde

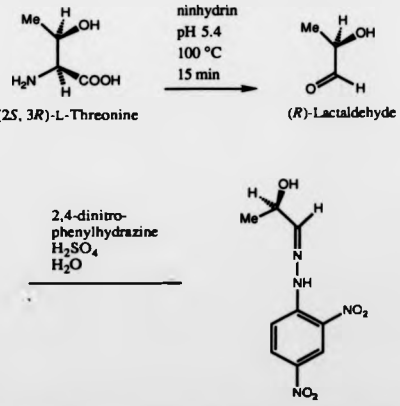


**Figure 2.67** The enantiomeric separation of the 2,4-dinitrophenylhydrazones of (*R*)- and (*S*)-lactaldehyde


The 2,4-dinitrophenylhydrazones of synthetic (*S*)-lactaldehyde (peak 1) and (*R*)-lactaldehyde (peak 2) were resolved by chiral HPLC using a Chiralcel OB column eluted with 2-propanol-hexane (1:4, v/v) at a flow rate of 0.5 $cm^3\ min^{-1}$ (chromatogram A). The (*R*)-lactaldehyde derivative (chromatogram B) was co-injected with authentic racemate (chromatogram C).

The derivatives of the enzymically produced lactaldehyde were analysed by chiral HPLC [Figure 2.68 (co-injections with authentic racemate are not shown) and Table 2.12]. The results were highly reproducible and remarkably similar to those obtained with acetoin. Both enzymes produced lactaldehyde with poor to moderate optical purity. They produced

predominantly opposite enantiomers with absolute configurations consistent with those obtained for acetoin. The optical purity of lactaldehyde appeared not to be affected by either enzyme concentration, enzyme purity, the time of incubation or the presence of sodium 2,2-dimethyl-2-silopentane-5-sulfonate (DSS). Considering the fact that the optically pure synthetic material was derivatised and purified in an identical manner to the enzymic material, it was extremely unlikely that any racemisation had occurred during any of these processes.

**Figure 2.68** The determination of the optical purity of lactaldehyde produced by PDC using chiral HPLC


The 2,4-dinitrophenylhydrazone of the lactaldehyde produced by YPDC (chromatogram A) and ZMPDC (chromatogram B) were analysed by chiral HPLC using a Chiralcel OB column eluted with 2-propanol-hexane (1:4, v/v) at a flow rate of 0.5 $cm^3\ min^{-1}$.

**Table 2.12** The optical purity of the lactaldehyde produced by YPDC and ZMPDC[a]

| Enzyme (concentration/U $cm^{-3}$) | Enantiomeric excess (%)[b] |
|---|---|
| YPDC (5.9) | 22 (*R*) |
| Fractionated YPDC (3.4) | 19 (*R*)[c] |
| Fractionated YPDC (3.4) | 20 (*R*)[c] |
| ZMPDC (5.3) | 52 (*S*) |
| ZMPDC (6.1) | 56 (*S*) |
| ZMPDC (6.1) | 55 (*S*) |
| ZMPDC (6.1) | 61 (*S*)[c,d] |

[a]Reaction mixtures contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), sodium pyruvate (100 mmol $dm^{-3}$), glyoxylate (100 mmol $dm^{-3}$) and either ZMPDC (186 U $mg^{-1}$), YPDC (12 U $mg^{-1}$) or fractionated YPDC (23 U $mg^{-1}$). The mixtures were incubated at 30 °C for 24 h.

[b]Determined by chiral HPLC of the corresponding 2,4-dinitrophenylhydrazone derivative.

[c]DSS was omitted from the reaction mixture.

[d]The reaction mixture was incubated for 17 h.

**2.2.5** *The ZMPDC-catalysed formation of aromatic acyloins.*— ZMPDC catalysed the formation of PAC (phenylacetylcarbinol, 1-hydroxy-1-phenyl-2-propanone) from benzaldehyde and pyruvate. Fluoro- and chloro-PAC were also formed from the corresponding substituted benzaldehydes (Figure 2.69). The rate of formation of acetaldehyde and acetoin was not significantly affected by the addition of the aromatic aldehydes at a concentration of 20 mmol $dm^{-3}$. Acetoin was therefore the major product. However, the addition

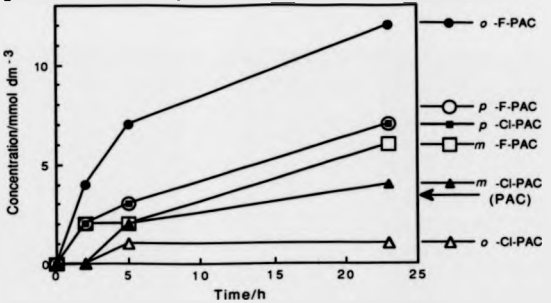
of the aromatic aldehydes at a concentration of 100 mmol $dm^{-3}$ inhibited the formation of acetaldehyde, acetoin and aromatic acetylcarbinol completely.

**Figure 2.69**


R = H, 2-F, 3-F, 4-F, 2-Cl, 3-Cl, 4-Cl

It can be seen from Figure 2.70 that the rate of formation of acyloin was dependent on the substitution of the benzaldehyde substrate. All of the fluoro- and chloro-benzaldehydes reacted faster than benzaldehyde, except for 2-chlorobenzaldehyde. In general, electron-withdrawing groups would be expected to activate benzaldehyde in a manner similar to the way they increase the acidity of benzoic acid. Substitution of benzoic acid at the 2 position by such groups causes a relatively larger increase in acidity, and this effect is called the *ortho*-effect. Thus, 2-fluorobenzaldehyde was indeed the most reactive compound. 2-Chlorobenzaldehyde was the least reactive substrate of all of those tested and was the only aromatic substrate which was less reactive than benzaldehyde. Therefore there appeared to be an additional factor which determined the reactivity of this compound. It is likely that substitution at the 2 position of benzaldehyde by the relatively large chloro group resulted in unfavourable binding of this substrate to the ZMPDC active site. It is possible that steric factors also contributed somewhat to the rate of reaction of the 3-substituted benzaldehydes.

**Figure 2.70** ZMPDC-catalysed formation of fluoro- and chloro-PAC

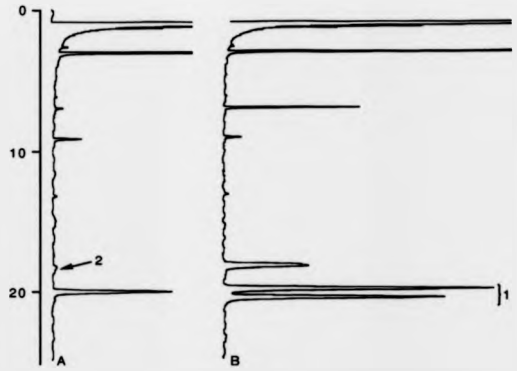



The reaction mixtures contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), sodium pyruvate (100 mmol $dm^{-3}$), aromatic aldehyde (20 mmol $dm^{-3}$) and ZMPDC (2.06 U) and were incubated at 30 °C. Product formation was monitored by 220 MHz $^1H$ NMR.

The optical purity of the aromatic acetylcarbinols was determined by chiral GC (Figure 2.71).** All of the aromatic acetylcarbinols were produced by ZMPDC in an optically pure form (Table 2.13). This was in sharp contrast to the poor to moderate optical purity of acetoin and lactaldehyde produced by this enzyme. In addition, small amounts of the benzoylmethylcarbinol isomers were detected. In general, the enantiomers of the isomers were not resolved using this column. However, the 2- and 4-fluoro substituted isomers were well resolved and it appeared that these were also optically pure. The production of these isomers in an optically pure form raises interesting questions about their formation.

** The optical purity of the aromatic acyloins was determined by Dr Vladimir Kren.

**Figure 2.71** The chiral analysis of aromatic acetylcarbinols produced by ZMPDC




In this example, the reaction mixture contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 µmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), sodium pyruvate (100 mmol $dm^{-3}$), 3-F-benzaldehyde (20 mmol $dm^{-3}$) and ZMPDC (2.06 U) and was incubated at 30 °C for 5 h. The enantiomers of 3-F-PAC (1) were resolved by chiral GC using a Lipodex A column at 93 °C (chromatogram A). There was a small peak indicating the presence of the benzoylmethylcarbinol isomer (2). The sample was co-injected with authentic racemic material (B). Retention time is shown in min.

**Table 2.13** The optical purity of aromatic acetylcarbinols produced by ZMPDC[a]

| Substrate | Acetylcarbinol enantiomeric excess (%)[b] | | |
|---|---|---|---|
| | 1 h | 5 h | 23 h |
| Benzaldehyde | >98 | >98 | >98 |
| 2-F-Benzaldehyde | >98 | >98 | >98 |
| 3-F-Benzaldehyde | >98 | >98 | 97 |
| 4-F-Benzaldehyde | >98 | >98 | >98 |
| 2-Cl-Benzaldehyde | >98 | >98 | >98 |
| 3-Cl-Benzaldehyde | >98 | >98 | >98 |
| 4-Cl-Benzaldehyde | >98 | >98 | >98 |

[a]The reaction mixtures contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), sodium pyruvate (100 mmol $dm^{-3}$), aromatic aldehyde (20 mmol $dm^{-3}$) and ZMPDC (2.06 U) and were incubated at 30 °C. Aliquots were extracted after 1, 5 and 23 h incubation.

[b]The optical purity of the aromatic acetylcarbinols was determined by chiral GC using a Lipodex A column.

The formation of acetoin from either pyruvate or acetaldehyde with ZMPDC was described above. Similarly, ZMPDC also catalysed the formation of aromatic acetylcarbinols from aromatic aldehydes and acetaldehyde. The rate of this reaction was several-fold lower than that in the presence of pyruvate. The products were all optically pure and of the same configuration as those obtained with pyruvate (Table 2.14). The absolute configurations of the aromatic acetylcarbinols were not known at this stage. The possibility that they may be the same could not be confirmed by their elution order from the

chiral column alone. However, the elution order of the products obtained with ZMPDC were identical to those obtained with YPDC and a recent study by Kren *et al.* has established that YPDC produces exclusively (*R*)-aromatic acetylcarbinols.[227] Therefore, ZMPDC also produced exclusively (*R*)-aromatic acetylcarbinols.

**Table 2.14** The optical purity of aromatic acetylcarbinols produced by ZMPDC with acetaldehyde[a]

| Substrate | Acetylcarbinol enantiomeric excess (%)[b] |
|---|---|
| Benzaldehyde | >98 |
| 2-F-Benzaldehyde | >98 |
| 3-F-Benzaldehyde | >98 |
| 4-F-Benzaldehyde | >98 |

[a]The reaction mixtures contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 $\mu$mol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), acetaldehyde (100 mmol $dm^{-3}$), aromatic aldehyde (20 mmol $dm^{-3}$) and ZMPDC (2.06 U) and were incubated at 30 °C for 48 h.

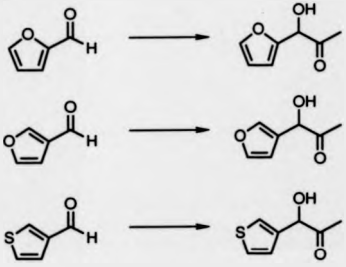
[b]The optical purity of the aromatic acetylcarbinols was determined by chiral GC using a Lipodex A column.

When aromatic aldehydes were incubated with ZMPDC in the presence of glyoxylate, there was no indication of the production of the corresponding aromatic formylcarbinols.

**2.2.6** *The ZMPDC-catalysed formation of heterocyclic acyloins.*— ZMPDC was found to catalyse the formation of acetylcarbinols from heterocyclic aldehydes (Figure 2.72). The order of reactivity, in descending order, was 3-

furaldehyde, 2-furaldehyde and 3-thenaldehyde. All of the heterocyclic substrates were considerably less reactive than benzaldehyde and 2-thenaldehyde did not produce any detectable amounts of product. It must be stated that the products were only tentatively identified by chiral GC. Presumably, both electronic and steric factors contributed to the relative reactivity of these compounds. The heterocyclic aldehydes were more inhibitory than the aromatic aldehydes.

**Figure 2.72**




The optical purity of the heterocyclic acetylcarbinols was determined by chiral GC (Table 2.15).** These products were not optically pure. However, it was found that these compounds racemised within a period of days despite being in the form of ethyl acetate extracts that were dried ($Na_2SO_4$) and stored at 4 °C. Therefore, it seemed likely that they were in fact produced by ZMPDC in an optically pure form.

** The optical purity of the heterocyclic acyloins was determined by Dr Vladimir Kren.

**Table 2.15** The optical purity of heterocyclic acetylcarbinols produced by ZMPDC[a]

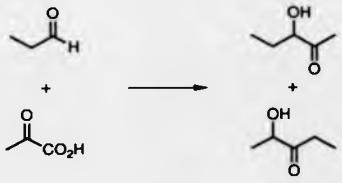
| Substrate (Concentration/mmol $dm^{-3}$) | Acetylcarbinol enantiomeric excess (%)[b] |
|---|---|
| 2-Furaldehyde (20) | 90 |
| 3-Furaldehyde (20) | 80 |
| 3-Thenaldehyde (15) | >90 |

[a]The reaction mixtures contained pH 6.0 sodium citrate buffer (100 mmol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 μmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$), acetaldehyde (100 mmol $dm^{-3}$), heterocyclic aldehyde and ZMPDC (2.06 U) and were incubated at 30 °C for 23 h.

[b]The optical purity of the heterocyclic acetylcarbinols was determined by chiral GC using a Lipodex A column.

**2.2.7** ***The ZMPDC-catalysed formation of acyloins from propanal.***— 2-Hydroxy-3-penatanone and 3-hydroxy-2-pentanone were produced when ZMPDC was incubated with sodium pyruvate (100 mmol $dm^{-3}$) and propanal (100 mmol $dm^{-3}$) (Figure 2.73). The products were formed in roughly equal amounts at a rate comparable with that observed for 2-furaldehyde. Despite the fact that propanal, acting as both donor and acceptor, was able to form acyloins it apparently did not condense with itself to form propionin (4-hydroxy-3-hexanone).

**Figure 2.73**



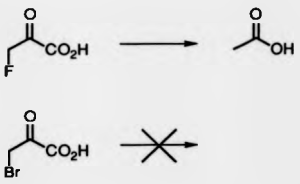
**2.2.8** ***Additional 2-oxo acids tested for their ability to form acyloins.***— 3-Hyroxypyruvate (3-hydroxy-2-oxopropanoate) was found to be quantitatively decarboxylated by ZMPDC to form glycolaldehyde (hydroxyethanal) at approximately one half of the rate of pyruvate decarboxylation (Figure 2.74). The identity of glycolaldehyde was confirmed by $^1$H NMR spectroscopy of a reaction mixture spiked with authentic material. Glycolaldehyde was not detected in control reactions without enzyme. The possibility of forming acyloin compounds with a number of aldehydes and 2-oxo acids was tested. No acyloin compounds were detected using TLC, GC and $^1$H NMR spectroscopy with either pyruvate, acetaldehyde, propanal, benzaldehyde, 2-fluorobenzaldehyde or glycolaldehyde.

**Figure 2.74**

3-Fluoropyruvate (3-fluoro-2-oxopropanoate) was decarboxylated by ZMPDC at approximately on tenth the rate with pyruvate. The product was not 2-fluoroethanal, but acetate (Figure 2.75). The identity of the product was confirmed by $^1$H NMR spectroscopy of a reaction mixture spiked with sodium acetate. Acetate was not detected in control reactions without enzyme. ZMPDC did not catalyse the formation of acyloins from 3-fluoropyruvate

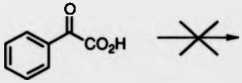
when incubated in the presence of either pyruvate or acetaldehyde. The possibility that nucleophilic compounds could be acetylated by ZMPDC in the presence of 3-fluoropyruvate was tested. Acetylation of either ethanol, hydroxylamine, glycine or serine was not detected. By contrast, 3-bromopyruvate (3-bromo-2-oxopropanoate) completely inactivated ZMPDC without any detectable turnover of this substrate.

**Figure 2.75**



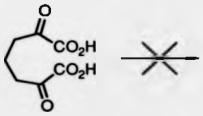

Oxophenylethanoate was not decarboxylated by ZMPDC (Figure 2.76). The formation of the benzoylmethylcarbinol from this 2-oxo acid and acetaldehyde was not detected by $^1H$ NMR spectroscopy.

**Figure 2.76**



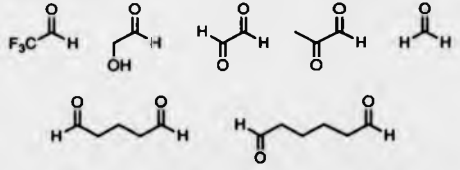

The ability of ZMPDC to catalyse the formation of cyclic acyloins from a di-2-oxo acid was tested with 2,6-dioxoheptanedioate. In addition, this substrate was incubated in the presence of pyruvate at a concentration which did not inhibit pyruvate decarboxylation. No acyloin compounds were detected by $^1H$ NMR spectroscopy in either the reaction mixtures or $CDCl_3$ extracts (Figure 2.77). It was not clear from these results as to whether the di-2-oxo acid was decarboxylated by ZMPDC.

Figure 2.77



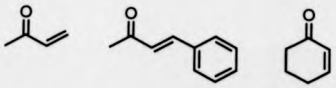
**2.2.9** *Additional aldehydes tested for their ability to form acyloins.*— A number of additional aldehydes were tested for their ability to form acyloins with either pyruvate or acetaldehyde. The di-aldehydes could in principle have formed either di-acyloins, cyclic acyloins or polymers. No acyloins were detected using $^1H$ NMR spectroscopy with either 2,2,2-trifluoroethanal, glycolaldehyde, 2-oxoethanal, 2-oxopropanal, formaldehyde, hexandial or glutaraldehyde (Figure 2.78). These aldehydes were used at concentrations (typically 20 mmol $dm^{-3}$) which did not inhibit the decarboxylation of pyruvate.

Figure 2.78



**2.2.10** *Michael acceptors.*— The Michael acceptors 3-buten-2-one, 4-phenyl-3-buten-2-one and 2-cyclohexanone did not give any products when incubated with pyruvate in the presence of ZMPDC (Figure 2.79). These compounds were added at concentrations (*circa* 20 mmol $dm^{-3}$) which did not inhibit pyruvate decarboxylation. $^1H$ NMR spectroscopy of $CDCl_3$ extracts of the reaction mixtures indicated that the Michael acceptors were unchanged.

**Figure 2.79**



## 2.3 Discussion

Recombinant ZMPDC was purified from recombinant *E. coli* to homogeneity and was shown to have identical characteristics to ZMPDC purified from the wild-type *Z. mobilis* strain using chromatographic and electrophoretic techniques. (Electrospray ionisation mass spectrometry is a powerful new technique which could be used to compare the molecular weight of the wild-type and recombinant enzymes to an accuracy of a few daltons.)[228] This is the first time that the purification and characterisation of the recombinant enzyme has been reported. The wild-type enzyme has been purified using different purification procedures to that described presently.[147,148,159] All of the procedures have used anion exchange chromatography as a key step in the purification of the enzymes. However a dye ligand chromatography step has been used by two groups[147,159] rather than a gel filtration step.

In the presently described procedure, there appeared to be components of molecular weight below 10 000 in the cell-free extracts which interfered with the binding of both wild-type and recombinant ZMPDC to anion exchange resins. This problem was overcome by subjecting the cell-free extracts to ultra-filtration before applying the protein to the anion exchange resin. The addition of surfactants to the cell-free extracts also alleviated this problem.[229] It is therefore possible that either hydrophobic or predominantly cationic low molecular weight material was binding to the relatively hydrophobic and anionic ZMPDC[192] and causing the enzyme not to bind to the resin efficiently. The use of surfactants to overcome such problems has been reported.[230] That this problem occurs with ZMPDC was not reported by other groups, presumably because purification steps, prior to the anion exchange step, removed these components.

The maximum specific activity of the purified wild-type enzyme that has been reported has been 181 U $mg^{-1}$ and this preparation was obtained in 23% overall yield.[148] The present method compares favourably with these results as recombinant enzyme of specific activity 150 U $mg^{-1}$ in 14% overall yield was obtained without any attempt to optimise the procedure. The present procedure has since been improved and material of specific activity 186 U $mg^{-1}$ has been obtained in a similar yield.[229] This is the highest specific activity of ZMPDC thus far reported. Other groups have reported good to excellent yields (75—88%) of the wild-type enzyme at the expense of the purity of the enzyme (120-134 U $mg^{-1}$).[147,159]

The amino acid sequences of ZMPDC and YPDC are homologous[193,195] and share 27% identity and 16% similarity (Figure 2.80). They also share a putative TDP binding sequence motif common to all known TDP dependent enzymes.[197] YPDC has been shown to be isolated in either a homo- or hetero-tetrameric form (see section 2.1.4.7). The commercial YPDC preparation used in the present study appeared to be of the homotetrameric form, and therefore it is assumed that the sequence of this enzyme is as described by Kellermann *et al.*[189]

**Figure 2.80** Sequence alignment of YPDC and ZMPDC

```
ZM  SY--TVGTYLAAL--QIGLKHHFAVAGDYNLVLLDNLLLNKNMECVYCCN  46
Y   MSEITLGKYLFERLKQVNVNTVFGLPGDFNLSLLDKIYEVEGMRWAGNAN  50

ZM  ELNCGFSAEGYARAKADAAAVVTYSVGALSAFDAIGGAYAENLPVILISG  96
Y   ELNAAYAADGYARIKGMSCIITTFGVGELSALNGIAGSYAEHVGVLHVVG  100

ZM  APNNNDHAAGHVLHHALGKTDYHYQLEMAKNITAAAEAIYTPEEAPAKID  146
Y   VPSISSQAK-QLLLHTLGNGDFTVFHRMSANISETTAMITDICTPQAEID  149

ZM  HVIKTALREKKPVYLEIACNIASMPCAAPGPASALFNDEASDEASLNAAV  196
Y   RCIRTTYVTQRPVYLGLPANLVDLNVPAKLLQTPIDMSLKPNDAESEKEV  199

ZM  EETLKFIANRDKVAVLVGSKL--RAAGAEEAAVKFADALGGAVATMAAAK  244
Y   IDTILVLVKDAKNPVILADACCSRHDVKAETKKLIDLTQFPAFVTPMGKG  249

ZM  SFFCKKTALHRYLMGEVSYPGVEKTMKEADAVIALAPVFNDYSTTGWTDI  294
Y   SISEQHPRYGGVYVGTLSKPEVKEAVESADLILSVGALLSDFNTGSFSYS  299

ZM  PDPKKLVLAEPRSVVVNGVRFPSVHLKDYLTRLAQKVSKKTGALDFFKSL  344
Y   YKTKNIVEFHSDHMKIRNATFPGVQMKFVLQKLLTNIADAAKG---YKPV  346

ZM  NAGELKKAAPADPSAPLVNAEIAR-QVEALLTPNTTVIAETGDSWFNAQR  393
Y   AVPARTPANAAVPASTPLKQEWMWNQLGNFLQEGDVVIAETGTSAFGINQ  396

ZM  MKLPNGARVEYEMQWGHIGWSVPAAFGYAVGA----PERRNILMVGDGSF  439
Y   TTFPNNTYGISQVLWGSIGFTTGATLGAAFAAEEIDPKKRVILFIGDGSL  446

ZM  QLTAQEVAQMVRLKLPVIIFLINNYGYTIEVMIHD--GPYNNIKNWDYAG  487
Y   QLTVQEISTMIRWGLKPYLFVLNNDGYTIEKLIHGPKAQYNEIQGWDHLS  496

ZM  LMEVFNGNGGYDSGAGKGLKAKTGGELAEAIKVALANTDGPTLIECFIGR  537
Y   LLPTFGAK---DYETHRVATTGEWDKLTQD--KSFNDNSKIRMIEVMLPV  541

ZM  EDCTEELVKWGKRVAARQQP                                557
Y   FDCSTKLG                                            549
```

The sequences of YPDC (Y; *S. cerevisiae*)[189] and ZMPDC (ZM; *Z. mobilis* ATCC 31821)[192] were aligned using PC Gene (6.5; IntelliGenetics Inc., California, US). The putative TDP binding sequence motif[197] is underlined. Sequence identity is indicated by "|" and sequence similarity is indicated by "s".

Given the fact that the two enzymes also have similar quaternary structures, it seems likely that they have similar secondary and tertiary structures. YPDC has been crystallised and the X-ray structure is currently being elucidated by Dyda *et al.*[201] Electron microscopy of YPDC reveals that the subunits of this enzyme appear to be rod-shaped molecules which form either "X"- or "Y"-shaped dimers.[199] These dimers presumably dimerise to form the active tetramers.

Acetoin optical purity was found to be measured conveniently by chiral GC using a Lipodex A column without the need for derivatisation and without any indication of racemisation during analysis (such chiral GC columns have been reviewed recently).[231] YPDC was found to catalyse the formation of acetoin from either pyruvate, pyruvate and acetaldehyde or acetaldehyde alone with an optical purity of 46 ± 1, 46 ± 1 and 44 ± 4% ee, respectively. This value of ee is in good agreement with that reported previously (54% ee).[137] It was assumed that the absolute configuration of the predominant isomer was (*R*) as reported by Chen and Jordan.[137] Chen and Jordan's finding that the optical purity of acetoin formed from acetaldehyde alone appeared to be only 27% ee was not confirmed. Presumably, their optical rotation measurements were affected by optically active contaminants.

Of interest was the observation that acetoin of optical purity 39 ± 2% ee was formed by YPDC from acetaldehyde alone in the presence of the allosteric activator pyruvamide. This value of ee may not be significantly different from that obtained with acetaldehyde in the absence of pyruvamide (44 ± 4% ee). However it appears to be significantly lower than that obtained in the presence of pyruvate (46 ± 1% ee). The allosteric activation of YPDC in known to lead to conformational changes of the active site of the enzyme[175] such that the enzyme is converted from an inactive form to an active form.[169,170] It is possible that the conformational changes manifested by

pyruvate are not qualitatively identical to those manifested by either pyruvamide or acetaldehyde. This could account for the depressed optical activity of acetoin produced by YPDC in the absence of pyruvate. It must be emphasised that this effect is rather small and further investigation would be required to confirm this result. However, there is some precedent for this type of effect. The relative amounts of *p*-methylcinnamaldehyde and *p*-methyldihydrocinnamic acid produced by YPDC from (*E*)-4-(4-tolyl)-2-oxo-3-butenoic acid were affected by the addition of pyruvamide (see section 2.1.4.7).[180]

In addition, acetoin was produced from acetaldehyde alone and in the presence of pyruvamide at approximately four times the rate obtained in the absence of pyruvamide. This would suggest that pyruvamide caused quantitatively and/or qualitatively different conformational changes of YPDC as compared with acetaldehyde. There is only one other report of the effect of pyruvamide on a YPDC catalysed reaction other than the decarboxylation of aliphatic 2-oxo acids. The rate of (*E*)-4-(4-tolyl)-2-oxo-3-butenoic acid turnover was increased up to 50-fold by the presence of pyruvamide.[180] Therefore, it would be of interest to study the effect of pyruvamide on the rate of other YPDC catalysed acyloin condensations.

Given the information now available for YPDC, the two-site mechanism for acetoin synthesis, proposed by Juni[134] in 1961 (see section 2.1.4.4), may be reinterpreted. There is no strong evidence for two distinct sites for the binding of active acetaldehyde to the active site of YPDC. However, there is a significant amount of evidence for the role of conformational changes in YPDC-mediated catalysis (see section 2.1.4.7). Therefore, it would seem reasonable to suggest that instead of there being two distinct sites that are capable of binding active acetaldehyde there are in fact two conformational states that the enzyme can adopt when active acetaldehyde is bound to the

enzyme. The finding that YPDC can be treated with yeast proteases such that the acetoin forming activity of the enzyme is maintained and the acetaldehyde forming activity is lost[135,136] may indicate that such limited proteolysis may affect the conformation of the enzyme rather than alter the second site. It must be stated that there is no direct evidence for either interpretation of the two-site mechanism.

The optical purity of acetoin produced by ZMPDC from pyruvate was 28 ± 3% ee. This value of ee appeared to be independent of the substrates and the presence of pyruvamide. This enzyme is known not to exhibit allosteric kinetics[147,159] and thus pyruvamide did not affect the rate of acetoin production from acetaldehyde alone with ZMPDC. Surprisingly, the absolute configuration of the predominant enantiomer was the opposite to that produced by YPDC and identical[127,128,138] to that produced by the wheat germ enzyme. Therefore, the prokaryotic ZMPDC may share a greater similarity to wheat germ PDC than to the eukaryotic YPDC. In addition, ZMPDC may be in general more closely related to plant PDCs than to YPDC because acetoin isolated from plant meals is predominantly of the (*S*) configuration.[3]

Davies and Corbett tentatively identified lactaldehyde as a product obtained from glyoxylate and acetaldehyde with wheat germ PDC.[153,154] In the present study, lactaldehyde was unambiguously identified as the product formed from glyoxylate and acetaldehyde by either YPDC or ZMPDC. These enzymes produced lactaldehyde with poor to moderate optical purity with the predominant enantiomers having absolute configurations consistent with those obtained with acetoin. The optical purity was determined by chiral HPLC of the corresponding 2,4-dinitrophenylhydrazone of lactaldehyde using a Chiralcel OB column. Lactaldehyde is known to undergo racemisation in acidic conditions at high temperatures.[232] Care was taken not to expose

lactaldehyde to temperatures above 25 °C and racemisation was not detected during the derivatisation and analytical procedures.

The formation of lactaldehyde by either ZMPDC or YPDC has not been reported previously. Lactaldehyde and a number of other aliphatic acyloins have been tentatively identified as products of wheat germ PDC using glyoxylate as the donor.[153,154] It is possible that ZMPDC and YPDC are also capable of forming aliphatic acyloins other than lactaldehyde from glyoxylate.

Bringer-Meyer and Sahm reported that ZMPDC catalysed the formation of PAC from pyruvate and benzaldehyde.[10] In the present work, purified ZMPDC was shown to produce aromatic acetylcarbinols in optically pure forms from unsubstituted and fluoro- and chloro-substituted benzaldehydes. Recently, YPDC has also been shown to catalyse the formation of these compounds in optically pure forms.[233] Since *S. cerevisiae* produced (*R*)-PAC and YPDC produces PAC with the same absolute configuration, as determined by chiral GC, YPDC also produced (*R*)-PAC.[233] Circular dichroism spectroscopy of the products obtained with YPDC and yeast indicate that all of these aromatic acetylcarbinols share the same absolute configuration.[227] Comparison of the products obtained with YPDC and ZMPDC by chiral GC showed unambiguously that both YPDC and ZMPDC produce (*R*)-aromatic acetylcarbinols.

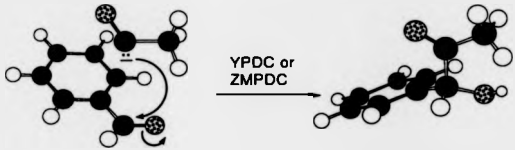
There is a significant amount of evidence that the same catalytic site is responsible for pyruvate decarboxylation and acyloin synthesis with YPDC (see section 2.1.4.7). It is therefore likely that this is also the case for ZMPDC. It has been established that PDC was responsible for the formation of the acyloins described in the present investigation and that no racemisation of the acyloin products was observed in the reaction conditions and in the determination of the optical purity of the products. Since the YPDC and

ZMPDC used in this study were both homotetramers, the optical purity of the products obtained must have been determined by the architecture of their active sites.

The production of acyloins by ZMPDC and YPDC with radically different optical purities and predominant optical purities can be explained by the orientation of the binding of the acceptor aldehydes to the active site. Both enzymes catalyse the formation of PAC from benzaldehyde and active acetaldehyde, derived from pyruvate, with an (*R*)-absolute configuration in an optically pure form. Therefore, benzaldehyde must bind exclusively in such an orientation to the active site of both enzymes such that active acetaldehyde attacks the *si*-face of benzaldehyde (Figure 2.81). One can speculate that the carbonyl group of the acceptor aldehyde benzaldehyde would require precise orienting relative to active acetaldehyde to allow the formation of the new carbon-carbon bond. In addition, it is most likely that there is a single acceptor binding site, say on one side of the TDP molecule. The suggestion that tryptophan residues almost certainly cover most of one side of the coenzyme of YPDC is consistent with this hypothesis.[145]

Given these assumptions, there must be a relatively large hydrophobic binding pocket, which can accommodate the phenyl group, and a relatively small binding pocket which can accommodate the aldehydic hydrogen. Since the small binding pocket can not accommodate the phenyl group, benzaldehyde can bind to the active site of these enzymes in only one orientation. The same arguments would have to apply for the binding of the TDP-diol intermediate, which precedes the liberation of free PAC.

**Figure 2.81** Schematic illustration of the stereochemistry of PAC formation by YPDC and ZMPDC




YPDC catalysed the formation of (*R*)-acetoin with an optical purity of 46% ee from pyruvate. The acceptor aldehyde acetaldehyde must bind predominantly to the active site of YPDC in the same orientation as benzaldehyde (Figure 2.82). However, it would appear that acetaldehyde is able to bind in the opposite orientation indicating the that the small binding pocket is, to some extent, able to accommodate the methyl group as well as the aldehydic hydrogen.

By contrast, ZMPDC catalysed the formation of (*S*)-acetoin with an optical purity of 28% ee from pyruvate (Figure 2.83). Therefore, the small binding pocket is capable of binding the methyl group more favourably than the large binding pocket with ZMPDC. This is indeed a surprising result as ZMPDC and YPDC are structurally such similar enzymes that one would not expect there to be such a difference in the architecture of their active sites.

**Figure 2.82** Schematic illustration of the stereochemistry of acetoin formation by YPDC


**Figure 2.83** Schematic illustration of the stereochemistry of acetoin formation by ZMPDC


YPDC and ZMPDC catalysed the formation of lactaldehyde from acetaldehyde and active formaldehyde, derived from glyoxylate, with a mean optical purity of 20 (*R*) and 56 (*S*) % ee, respectively. These optical purities are broadly similar to those obtained with the formation of acetoin. Closer examination of these results reveals a fascinating insight into the structure of the active sites of these enzymes. YPDC produces 73% of the (*R*) enantiomer of acetoin and 60% of the (*R*) enantiomer of lactaldehyde, the difference between these

figures being 13% in favour of the (*S*) enantiomer. ZMPDC produces 36% of the (*R*) enantiomer of acetoin and 22% of the (*R*) enantiomer of lactaldehyde, the difference between these figures being 14% in favour of the (*S*) enantiomer. The stereochemistry of acyloin formation by both enzymes was therefore dependent on the nature of the donor and the acceptor.

The interpretation of this finding must involve the the effect of the substitution of the methyl group of active acetaldehyde with the hydrogen of active formaldehyde. The structure of the active aldehyde must therefore directly or indirectly affect the architecture of the active sites of both enzymes in an identical manner, despite the fact that the active sites of these enzymes are not identical in every respect. The observation that active formaldehyde formed by either enzyme was not capable of forming acyloin compounds with aromatic aldehydes is consistent with this hypothesis.

Propanal was found to act as both donor and acceptor in the ZMPDC catalysed reaction. The corresponding aliphatic acetylcarbinols formed with acetaldehyde were identified. However, the optical purity of the products was not determined. It is likely that with propanal acting as an acceptor, 3-hydroxy-2-pentanone was formed with an optical purity that was similar to that obtained with acetoin except that the (*R*) enantiomer would be more predominant (Figure 2.84). In other words, the ethyl group of propanal would presumably bind relatively more favourably to the large binding pocket than the methyl group of acetaldehyde. With propanal acting as a donor (2-oxobutanoic acid was also found to be a suitable donor[226]), a shift towards the production of the (*R*) enantiomer would also occur if the trend observed for active formaldehyde and acetaldehyde continued with active propanal (Figure 2.85).

**Figure 2.84** Optical purity of acyloins produced with pyruvate as the donor using ZMPDC

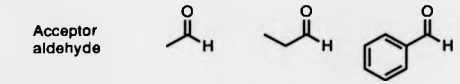
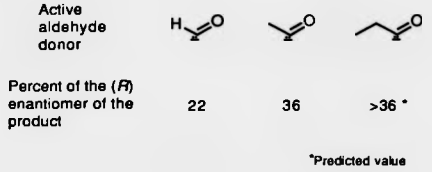



**Figure 2.85** Optical purity of acyloins produced from the acceptor acetaldehyde and active aldehydes using ZMPDC




If one considers the kinetics of the formation of acetoin by YPDC, then the optical purity of the product is a function of the relative rates of the formation of each enantiomer of the product. If we assume that the formation of each enantiomer obeys Michaelis-Menten kinetics then the relative rate of the formation of (*R*)- and (*S*)-acetoin is a function of their apparent $K_m$ and/or $V_{max}$ values. In one extreme case, the apparent $V_{max}$ values would be identical and the apparent $K_m$ values would be different.

Using the equation

$$\frac{N^s}{N^r} = e^{-\Delta G / RT}$$

the difference in the free energy of binding (where $\Delta G = \Delta G^\circ$) of acetaldehyde in the two binding modes can be estimated from

$$\Delta G = RT \ln (N^s / N^r)$$

For example, (*R*)-acetoin is produced by YPDC with an optical purity of 46% ee. This means that $N^s/N^r = 27/73$ and therefore, $\Delta G = 2.5$ kJ mol$^{-1}$. Similarly, ZMPDC produces (*S*)-acetoin with an optical purity of 28% ee, which gives $\Delta G = 1.4$ kJ mol$^{-1}$. In the other extreme case, where the apparent $K_m$s would be identical and the apparent $V_{max}$s would be different, the values of $\Delta G$ would reflect the differential free energy barrier ($\Delta G^\ddagger$) of the formation of each enantiomer of the product. These values of $\Delta G$ are rather small, indicating that the differences between the active sites of the enzymes are also small.

A similarity between the two enzymes was found. As well as the electronic effects of fluoro and chloro substitution, there appeared to be steric factors affecting the rate of aromatic aldehyde transformation. For example, 2-fluorobenzaldehyde was the most reactive aromatic aldehyde and 2-chlorobenzaldehyde was the least reactive with ZMPDC. Similar results have been obtained with YPDC.[233] Relatively large substituents at the 2 position of benzaldehyde, such as a chloro or a methoxy group, drastically reduced the rate of transformation by YPDC.[233] Thus, one can conclude that there may be a narrow "collar" in which the aldehydic group must fit in order for the acceptor aldehyde to bind correctly in the active sites of both enzymes. The observation that the substitution and geometry of the 2-carbon-carbon

double bond of α,β-unsaturated aldehydes affected that rate of acyloin condensations by yeast cells[49,51,54-56] in a similar manner is consistent with this conclusion if one assumes that PDC is the enzyme responsible for these biotransformations.

The heterocyclic aldehydes were relatively poor substrates of ZMPDC. The products obtained were essentially optically pure. Similar results were obtained with YPDC.[17,233] Considering the stereochemistry of the reactions discussed above it would seem very likely that both enzymes produced the (*R*)-heterocyclic acetylcarbinols.

ZMPDC did not catalyse the formation of either mono-acyloins or di-acyloins from di-aldehydic aldehydes. It is likely that despite the finding that the active site of ZMPDC is less lipophilic than that of YPDC,[10] these relatively hydrophilic substrates do not bind favourably to ZMPDC active site. In addition, cyclic acyloins were not formed from either di-aldehydes or di-2-oxo acids. Presumably, the active site of ZMPDC can not accommodate these substrates for steric reasons. Indeed, it has been suggested that the TDP of YPDC is almost completely buried in the enzyme molecule and that there is a narrow access for substrates to the coenzyme.[234]

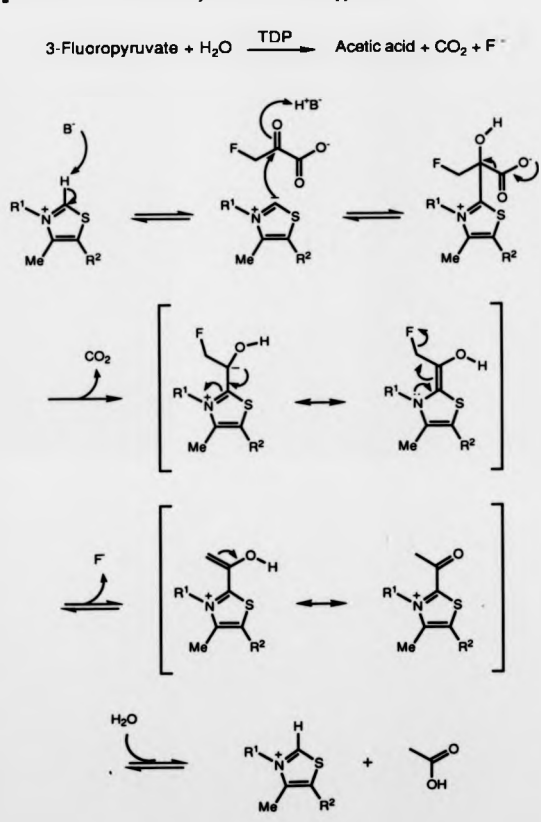
ZMPDC was found quantitatively to decarboxylate 3-hydroxypyruvate to glycolaldehyde. Recently, Thomas *et al.* have also described this decarboxylation reaction with ZMPDC.[235] Surprisingly, no acyloin products were formed with various aliphatic and aromatic aldehyde acceptors. Therefore, ZMPDC was not found to catalyse the transfer of active 2-hydroxyacetaldehyde in a manner analogous to transketolases. In addition, glycolaldehyde was not a suitable acceptor of active acetaldehyde.

3-Fluoropyruvate was not a suitable donor for ZMPDC-catalysed acyloin condensations either. In any case, this 2-oxo acid was found to be decarboxylated to acetic acid rather than 2-fluoroacetaldehyde. Similar results have been described by Gish *et al.* for the YPDC-catalysed reaction.[236] A mechanism was proposed where a fluoride ion is released from the active 2-fluoroacetaldehyde intermediate resulting in the formation of acetyl-TDP (Figure 2.86).[237]

The decarboxylation of 3-fluoropyruvate to acetate by a TDP dependent enzyme was first described by Leung and Frey.[237] The PDC component ($E_1$) of *E. coli* PDH (pyruvate dehydrogenase) was found to catalyse this reaction and a very similar reaction with pyruvate in the presence of the oxidant ferricyanide. Other groups have reported the YPDC-dependent oxidative decarboxylation of pyruvate in the presence of an oxidant.[238,239]

Although all of these studies identified acetic acid as the sole product, the enzymes were also found to be irreversibly inactivated by the acetylation of a cysteine residue at or near the active sites of the enzymes.[117,238,240] 3-Bromopyruvate was found to be a more potent irreversible inactivator of PDH[241,242] and ZMPDC (this study) than 3-fluoropyruvate.

**Figure 2.86** The decarboxylation of 3-fluoropyruvate



Huebner *et al.* found that acetaldehyde, which acts as in inhibitor of normal pyruvate decarboxylation, did not influence the rate of the oxidative decarboxylation of pyruvate.[239] This finding is consistent with the lack of acyloins formed by ZMPDC from 3-fluoropyruvate. Gruys *et al.* synthesised 2-acetyl-TDP and found that it was capable of non-enzymically acetylating several nucleophiles, such as hydroxylamine.[243] However, ZMPDC did not acetylate nucleophiles in the presence of 3-fluoropyruvate. It is possible that such acetylation reactions are possible in low water systems.

The yeast-mediated Michael addition reactions with 2,2,2-trifluoroethanol and α,β-unsaturated carbonyl compounds[86] can be interpreted in terms of the oxidation of trifluoroethanol to fluoral, the formation of active fluoral and the addition to α,β-unsaturated carbonyl compounds (see section 2.1.2). It was of interest to determine whether ZMPDC could catalyse such Michael additions. Fluoral was not found to be a suitable donor in ZMPDC-catalysed acyloin condensations, presumably because the hydration equilibrium of aqueous fluoral is such that fluoral exists essentially in the completely hydrated form. The observation that formaldehyde was not a suitable donor either is consistent with this interpretation. Therefore several Michael acceptors were incubated with ZMPDC and pyruvate rather than fluoral. ZMPDC was not found to catalyse Michael additions under these conditions. Although these experiments were not conducted with YPDC, it seems likely that enzymes other than PDC are involved with the yeast-mediated reactions.

One can conclude that many of the acyloin compounds formed by yeast cells can be synthesised with isolated PDCs. It remains to be seen whether this enzyme is responsible for all of the yeast mediated acyloin condensations. It would be of interest to use the recombinant *E. coli* as a whole-cell biocatalyst in acyloin condensations because of its very high PDC specific activity. It is possible that the use of the recombinant cells would have advantages over

the use of yeast cells in terms of the reaction rate of the desired reaction and the extent of competing side-reactions.

The ZMPDC-catalysed formation of acyloin compounds has some clear advantages over the use of yeast cells. Perhaps the main advantage is the possible use of acetaldehyde as a donor rather than pyruvate. This also makes the use of ZMPDC more attractive than the use of YPDC. In addition, the high thermostability of ZMPDC could allow this enzyme to be used at elevated temperatures. Furthermore, ZMPDC has a higher specific activity than YPDC and can be prepared on a large scale from recombinant *E coli*.

The isolated enzymes are also amenable to immobilisation, which would in principle allow the easy recovery and repeated re-use of the biocatalyst. A further advantage is the lack of side reactions encountered with the isolated enzymes. For example, there are no redox transformations of the substrates and products and also a lower incidence of product isomerisation and racemisation.

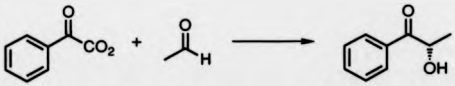
An intriguing observation was the highly purified ZMPDC-catalysed formation of the isomers of aromatic acetyl carbinols in an optically pure form. Similar observations have been made with YPDC.[227] These aromatic methylcarbinols are formed in very small quantities. It has been proposed that such an isomerisation of PAC can be catalysed by yeast cells (see section 2.1.1 and Figure 2.8).[40] However, the possibility that the both the ZMPDC and YPDC preparations contained a stereospecific acetylcarbinol dehydrogenase, a diol oxidase and the requisite cofactors is extremely remote. Therefore, the PDCs must be responsible for the formation of these isomers.

There is no possibility for the isomers to be derived from PAC through a purely TDP-dependent mechanism. It is possible that the methyl carbinols

could be formed from benzaldehyde, acting as the donor, and acetaldehyde, acting as the acceptor. However, 2-phenyl-2-oxoethanoic acid was not decarboxylated by ZMPDC and did not form the corresponding acyloin compounds.

Wilcocks and Ward have reported that the benzoylformate (oxophenylethanoate) decarboxylase of *Pseudomonas putida* catalysed the formation of (*S*)-2-hydroxy-1-phenyl-1-propanone (Figure 2.87) with 91-92% ee from acetaldehyde and benzoylformate.[244] This enzyme is involved in the catabolism of aromatic compounds in the mandelate pathway. It is unlikely that this enzyme is present in either *S. cerevisiae*, *Z. mobilis* or *E. coli* and extremely unlikely that this enzyme co-purifies with the PDC from these sources. Therefore, the apparent side reaction of PDC requires further investigation in order to account for the mechanism.

**Figure 2.87** The benzoylformate decarboxylase-catalysed acyloin condensation




The physiological role of PDC-catalysed acyloin condensations is not clear. However, both YPDC and ZMPDC can be used to form a number of optically pure acyloin compounds which are of value as chiral synthons. For example, (*R*)-PAC, the commercially valuable precursor of (-)-ephedrine, could be synthesised with the isolated enzymes rather than with yeast cells. Although YPDC and ZMPDC appear to have many properties in common, some very subtle differences have been discovered which imply some variation in the architecture of their active sites. A more complete understanding of these

differences requires the elucidation of their three-dimensional structures, which is eagerly awaited. It may be possible to model the binding of acetaldehyde in the two binding modes with the crystal structure of one of these enzymes. However, the differential energies are perhaps too low to obtain reliable results. When the crystal structures of both enzymes become available, more reliable modelling experiments will be possible as the enzymes can be directly compared and contrasted. This is perhaps a unique opportunity to model enzyme stereochemistry.

## 2.4 Experimental

**2.4.1** *General Methods*— Sodium pyruvate, sodium glyoxylate, aldehydes, NADH, TDP (cocarboxylase), ampicillin, L-threonine, pyruvate decarboxylase from brewer's yeast (YPDC), yeast alcohol dehydrogenase and proteins for gel filtration chromatography calibration were purchased from Sigma Chemical Co. Ltd. Acetaldehyde was distilled before use. Acetoin (dimer) was purchased from Fluka. Sodium 2,2-dimethyl-2-silopentane-5-sulfonate (DSS) was purchased from Aldrich. Yeast extract and agar were purchased from β-Laboratories and Difco Bacto, respectively. *Zymomonas mobilis* strain CP4 ATCC 31821 and the plasmid pLOI295,[223] harbouring the the genes for PDC and alcohol dehydrogenase II from this *Z. mobilis* strain, were obtained from Lonnie Ingram, University of Florida, USA. The Lipodex A chiral GC column was obtained from W. A. König, University of Hamburg, Germany. Electrophoresis molecular weight and isoelectric point (pI) calibration kits were obtained from Pharmacia LKB Biotechnology. Flash chromatography grade silica gel (mesh 230-400) and TLC plates (Kieselgel 60) were purchased from Merck Ltd. All other chemicals, whose sources are not cited, were of the highest quality available. Light petroleum refers to petroleum ether (b.p. 40-60 °C). Protein separations were carried out using the PhastSystem and FPLC equipment, respectively, from Pharmacia LKB Biotechnology. Analytical and preparative high pressure liquid chromatography (HPLC) separations were carried out using Gilson 302 and 305/306 solvent delivery systems, respectively. GLC was carried out using a Shimadzu GC-14A gas chromatograph and a Shimadzu Chromatopac C-R5A integrator. NMR spectra were obtained using Perkin Elmer 220 MHz R34, Bruker 250 MHz AFC250 and Bruker 400 MHz WH400 spectrometers. IR spectra were obtained using a Perkin Elmer 1720X spectrometer. Mass spectra were determined using a Kratos MS80 spectrometer. Spectrophotometric enzyme and protein assays were monitored using a Pye Unicam SP1800 spectrophotometer and scanning

measurements were obtained using a Philips PU8720 spectrophotometer. NMR coupling constants ($J$) are cited in Hz. Melting points were uncorrected. TLC plates were developed with a solution of tetrazolium blue (0.25%, w/v) in methanol-water (1:1, v/v) containing NaOH (3 mol $dm^{-3}$). Acyloin compounds appeared purple on a pale yellow background.

**2.4.2** *Bacterial Strains, Plasmids and Growth Conditions.*— *Escherichia coli* strain DH1 (with no detectable endogenous PDC activity) was transformed* with pLOI295,[223] harbouring the the genes for *Zymomonas mobilis* strain CP4 ATCC 31821 PDC and alcohol dehydrogenase II, using the method described by Chung *et al.*[245] Recombinants were selected* for resistance to ampicillin and for the expression of the *Z. mobilis* genes on aldehyde indicator plates, containing pararosaniline, as described by Ingram *et al.*[223] Transformed *E. coli* cells were grown at 37 °C in Luria broth[246] containing glucose (2 g $dm^{-3}$) and ampicillin (0.1 g $dm^{-3}$). Wild-type *Z. mobilis* ATCC 31821 was grown at 30 °C in broth containing yeast extract (10 g $dm^{-3}$), $KH_2PO_4$ (0.3 g $dm^{-3}$) and glucose (2 g $dm^{-3}$). Solid media contained agar (15 g $dm^{-3}$) and glucose (20 g $dm^{-3}$). Cells were prepared on a gram scale from anaerobic 2 and 20 $dm^{-3}$ fermentation broths using 10% (v/v) inocula.

**2.4.3** *Assay of PDC Activity.*— The PDC activity was determined essentially by the method of Ullrich.[212] The enzyme was diluted with pH 6.0 sodium citrate buffer (0.1 mol $dm^{-3}$) containing TDP (1 mmol $dm^{-3}$) and $MgCl_2$ (10 mmol $dm^{-3}$) prior to activity determination. The assay mixture (3.0 $cm^3$) contained pH 6.0 sodium citrate buffer (2.7 $cm^3$; 0.1 mol $dm^{-3}$), NADH + $H^+$ (100 $mm^3$; 6 mmol $dm^{-3}$), alcohol dehydrogenase (30 U) and a limiting amount of PDC solution (100 $mm^3$). Reactions were started by the addition of sodium pyruvate (100 $mm^3$; 0.3 mol $dm^{-3}$). The decrease in absorbance at 340

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* The transformation and selection of recombinants was performed by Margaret M. Turner.

nm was monitored at 30 °C. One unit (U) of enzyme activity was defined as the formation of one μmol of acetaldehyde per min. Protein was determined using the Bio-Rad Protein Assay.

**2.4.4** *Preparation of Enzymes.*— **2.4.4.1** *ZMPDC.* Transformed *E. coli* cells were harvested in the late exponential phase of growth by centrifugation, washed with pH 6.4 bistrispropane buffer (50 mmol $dm^{-3}$), containing TDP (1 mmol $dm^{-3}$) and $MgCl_2$ (1 mmol $dm^{-3}$), and resuspended in the same buffer. The cells were disrupted by three passes through a French pressure cell and cell debris was removed by centrifugation (39 200 × g; 4 °C; 30 min). The cell-free supernatant, containing the heat stable ZMPDC, was heat treated (60 °C; 10 min). Heat precipitated protein was removed by centrifugation (microcentrifuge). Material of molecular weight below 10 000 was diluted 5-fold by repeated ultrafiltration using Centriprep 10 (Amicon) devices and dilution with pH 7.0 bistrispropane buffer (20 mmol $dm^{-3}$) containing TDP (1 mmol $dm^{-3}$) and $MgCl_2$ (1 mmol $dm^{-3}$). The dilution of low molecular weight material was necessary for the efficient binding of the PDC to the ion exchange resin in the subsequent step. The ultrafiltration retentate (50 mg protein per injection) was applied to an FPLC Mono Q HR10/10 anion exchange column, previously equilibrated with pH 7.0 bistrispropane buffer (20 mmol $dm^{-3}$). The protein was eluted with a NaCl gradient (0—1 mol $dm^{-3}$) in the equilibration buffer at flow rate of 2 $cm^3$ $min^{-1}$. The maximum ZMPDC activity eluted with 0.11 mol $dm^{-3}$ NaCl. The pH of the active fractions was adjusted to 6.0 by adding pH 6.0 sodium citrate buffer (20%, v/v; 1.0 mol $dm^{-3}$) and dilute HCl (1 mol $dm^{-3}$). TDP and $MgCl_2$ were added to the active fractions to give 1 mmol $dm^{-3}$ and 10 mmol $dm^{-3}$ concentrations, respectively. Finally, the material (5 mg protein per injection) was applied to an FPLC Superdex 200 gel filtration column (330 $cm^3$ bed volume) previously equilibrated with pH 6.0 sodium citrate buffer (20 mmol $dm^{-3}$) containing NaCl (50 mmol $dm^{-3}$), TDP (1 mmol $dm^{-3}$) and $MgCl_2$ (10 mmol $dm^{-3}$). The

protein was eluted with the equilibration buffer at a flow rate of 3 $cm^3\ min^{-1}$. The elution volume of the maximum activity (157 $cm^3$) indicated that the enzyme had an apparent molecular weight of 185 000 ± 7 000 (lit., 200 000,[148] 240 000,[147] 219 700[159]). [Calibration proteins were: horse spleen ferritin (440 000 molecular weight; elution volume/$cm^{-3}$ 131), bovine liver catalase (232 000; 155), rabbit muscle aldolase (158 000; 160) and bovine serum albumen (68 000; 184)]. The purified ZMPDC appeared to be homogeneous, with a molecular weight of 245 000, on examination with native polyacrylamide gel electrophoresis (PAGE) with Coomassie Blue staining. The identity of the stained protein was confirmed by using the activity stain for PDC as described by Zehender *et al.* on a duplicate gel.[225] The duplicate gel was incubated in an aqueous solution (30 $cm^3$) of TDP (5 $mmol\ dm^{-3}$), $MgSO_4$ (5 $mmol\ dm^{-3}$), sodium pyruvate (30 $mmol\ dm^{-3}$) and sodium citrate (0.3 $mol\ dm^{-3}$; pH 6.0) at ambient temperature. After 0.25 h, an aqueous solution (450 $mm^3$) of 1,2-dianilinoethane (1.8 mg) containing glacial acetic acid (40%, v/v) was added. The gel was washed with distilled water after a further 25 min incubation. The subunit molecular weight of the enzyme was determined to be 65 000 (lit., 56 500,[148] 59 000[147]) by denaturing PAGE. The pI of the native enzyme was 5.00 (lit., 4.87[148]). The PDC was purified up to 5-fold and material of specific activity 150 and 186 $U\ mg^{-1}$ was obtained (lit.,[147,148] 120-181 $U\ mg^{-1}$).† ZMPDC was stable when stored at -20 °C with glycerol (50%, v/v). The wild-type ZMPDC purified by the above procedure from the *Z. mobilis* strain CP4 ATCC 31821 wild-type strain

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† Further quantities of purified cloned ZMPDC were prepared by Gregory Dean using the same method. Recently, the method was quickened by growing the transformed cells aerobically in a 6 $dm^{-3}$ fermenter and by using DEAE (diethylaminoethyl) fast-flow sepharose instead of Mono Q. In addition, *n*-dodecyl-β-D-glucopyranose, added to a concentration of 50% (w/w with respect to protein) allowed the enzyme to bind efficiently to the anion exchange resin without the need for the lengthy ultra-filtration step. Material of up to 186 $U\ mg^{-1}$ was obtained.

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exhibited identical chromatographic and electrophoretic properties to the recombinant enzyme.

**2.4.4.2** *YPDC* YPDC was used as supplied by Sigma (12 U $mg^{-1}$) unless stated otherwise. YPDC was purified by gel filtration chromatography[#] as described above except that the eluent contained dithiothreitol (0.5 mmol $dm^{-3}$), and that the material was completely dissolved in the minimum volume of the elution buffer before application to the column. The purified YPDC [23 U $mg^{-1}$ (lit.,[209,211,212] 80-85 U $mg^{-1}$)] appeared to be greater than 80% homogeneous on examination with denaturing PAGE with Coomassie Blue staining.

**2.4.5** *PDC-Catalysed Reactions.*— The analytical scale reaction mixtures contained pH 6.0 sodium citrate buffer (0.1 mol $dm^{-3}$), DSS (11.45 mmol $dm^{-3}$), TDP (15 µmol $dm^{-3}$), $MgSO_4$ (0.1 mmol $dm^{-3}$) and either recombinant ZMPDC (7.55 U; 150 U $mg^{-1}$) or YPDC (7.55 U; 12 U $mg^{-1}$). The use of different enzyme concentrations and enzyme specific activities are indicated in the results section. The substrates were added last, either directly or in the form of stock solutions dissolved in the sodium citrate buffer, to give a final reaction volume of 1 $cm^3$. The reaction mixtures were incubated at 30 °C. The product concentrations were determined by $^1H$ NMR (220 MHz) using DSS and citrate as internal standards. Aliquots (0.1 $cm^3$) were extracted with either ethyl acetate or diethyl ether (2 × 1 volume). The combined organic extracts were dried ($NaSO_4$) and submitted for TLC, GC and chiral analysis. Semi-preparative reactions (5 $cm^3$) were identical to the analytical scale reactions. The reaction mixtures were added to the 2,4-dinitrophenylhydrazine reagent as described below. The lactaldehyde derivative was purified by silica gel chromatography and HPLC as described

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[#] The purification of YPDC was performed by Gregory Dean and Nick Thomson.

below. The $R_f$ (TLC), $^1H$ NMR and mass spectra were indistinguishable from those obtained with authentic material.

**2.4.6** *GC Analysis of Acetoin.*— Acetoin was analysed by GC using a BP20 (25 m × 0.22 mm; film 0.25 μm; S.G.E. Ltd.). The injector, column and flame ionisation detector temperatures were 200, 72 and 250 °C, respectively. Using hydrogen as the carrier gas (linear flow rate/cm $s^{-1}$ 40), the retention time for acetoin was 6.8 min. Reaction mixtures were extracted with with ethyl acetate containing 2-heptanone (1:1500, v/v) as a internal standard. By extracting aqueous solutions of acetoin of known concentration a standard curve was determined which took into account the possibility of not extracting acetoin from reaction mixtures completely.

**2.4.7** *Chiral Analysis.*— **2.4.7.1** *Lactaldehyde.* The optical purity of lactaldehyde-2,4-dinitrophenylhydrazone was determined by HPLC [Chiralcel OB (25 cm × 4.6 mm), Baker, Daicel Chemical Industries Ltd.] with 2-propanol-hexane (1:4, v/v) as the eluent. Eluted compounds were detected by UV absorbance at 254 nm. (*RS*)-Lactaldehyde-2,4-dinitrophenylhydrazone (retention time/min 55 and 68) and (*R*)-lactaldehyde-2,4-dinitrophenylhydrazone (68) were eluted at a flow rate of 0.5 $cm^3$ $min^{-1}$. The lactaldehyde derivatives obtained from the preparative enzyme-catalysed reactions exhibited the same retention times as the synthetic material. Co-injections were performed with synthetic racemic material. The identical results were obtained when the eluted compounds were detected by absorbance at 350 nm.

**2.4.7.2** *Acetoin.* Acetoin optical purity was determined by chiral GLC [Lipodex A, OV1700 and 50% hexa-(2,3,6-*O*-pentyl)-α-cyclodextrin column (25 m × 0.25 mm)]. The injector, column and flame ionisation detector temperatures were 200, 20 and 250 °C, respectively. Using hydrogen as the carrier gas (linear flow rate/cm $s^{-1}$ 40), the retention times for the acetoin

enantiomers were 9.7 and 10.3 min. Assuming the major enantiomer produced by the YPDC is the same as that isolated by Chen *et al*[137], the elution order was (*R*)- and then (*S*)-acetoin. Acetoin obtained from the enzyme-catalysed reactions gave identical retention times to the racemate and co-injections confirmed the identity of the peaks.

**2.4.7.3** *Aromatic and heterocyclic acyloins.* The optical purity of aromatic and heterocyclic acyloins was determined[**] by chiral GC as described above, except that the column temperature was as indicated below. The enantiomers of the authentic racemates of PAC (retention time/min 44.72 and 45.65; column temperature 70 °C), *o*-fluoro-PAC (20.08 and 20.39; 78 °C), *m*-fluoro-PAC (19.40 and 19.93; 93 °C), *p*-fluoro-PAC (37.39 and 37.83; 78 °C), *o*-chloro-PAC (44.59 and 45.28; 85 °C), *m*-chloro-PAC (93.63 and 94.97; 85 °C), *p*-chloro-PAC (166.54 and 169.21; 78 °C), 2-furyl-PAC (15.19 and 15.58; 65 °C), 3-furyl-PAC (13.23 and 14.45; 75 °C) and 3-thenyl-PAC (51.36 and 51.77; 75 °C) were well resolved using this column. In each case, except for *o*-fluoro-PAC and *o*-chloro-PAC, the enantiomer of the biological product was the first enantiomer to elute from the column.

**2.4.8** *Lactaldehyde.*— (*RS*)- and (*R*)-Lactaldehyde were synthesised essentially by the method of Zagalak *et al.*[247] from DL- and L-threonine, respectively. A solution of threonine (0.3 g, 2.5 mmol) and ninhydrin (0.9 g, 5.0 mmol) in pH 5.4 sodium citrate buffer (60 $cm^3$; 50 mmol $dm^{-3}$) was boiled (100 °C; 15 min) and allowed to cool. The reaction mixture was filtered and Dowex 1-X8 ($HCO_3^-$) was added with stirring to pH 6.5. After 1.5 h the resin was removed by filtration. Dowex 50 ($H^+$) was added with stirring to the reaction mixture to pH 4.0. The resin was removed by filtration and the reaction mixture was concentrated under reduced pressure to between 5 and 10 $cm^3$.

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[**] The optical purity of aromatic and heterocyclic acyloins was determined by Dr Vladimir Kren. Enzymically produced material was co-injected with authentic racemic compounds.

The resin treatments were repeated on the concentrated reaction mixture resulting in a colourless solution of lactaldehyde containing some residual citrate. $\delta_H$(220 MHz, $H_2O$, DSS) 1.17 (3 H, d, *J* 6.7, Me), 3.69 (1 H, m, *CH*OH).

**2.4.9** *Lactaldehyde-2,4-dinitrophenylhydrazone*.— 2,4-Dinitrophenylhydrazine (0.25 g, 1.26 mmol) and then distilled water (5 $cm^3$) were added to concentrated sulfuric acid (5 $cm^3$) and the mixture was swirled until the reagent was dissolved. The resulting solution was diluted with distilled water (90 $cm^3$). Freshly prepared (*RS*)-lactaldehyde solution (3 $cm^3$) was added to the 2,4-dinitrophenylhydrazine reagent and the mixture was stirred at ambient temperature for 20 min. The resulting orange/yellow precipitate was filtered and washed with distilled water. The formation of the osazone derivative was not detected. The product was applied to a flash silica gel column and eluted with ethyl acetate-light petroleum (1:1, v/v). Pure product was obtained after preparative reverse phase HPLC [Dynamax 60A (8 μm $C_{18}$) column (25 cm × 10 mm) with guard column (5 cm × 10 mm), Rainin Instrument Co. Inc.] with an eluent of water-methanol (35:65, v/v) at a flow rate of 3 $cm^3\ min^{-1}$. Eluted compounds were detected by UV absorbance at 254 nm. Lactaldehyde-2,4-dinitrophenylhydrazone eluted at 12.1 min, m.p. 155-156 °C (from $CHCl_3$) (lit.,[248,249] 155-158 °C) (Found: $M^+$, 254.0658. $C_9H_{10}N_4O_5$ requires M, 254.0651); $\lambda_{max}$ (MeOH)/nm 354 (ε/$dm^3\ mol^{-1}\ cm^{-1}$ 6.4 × $10^6$), 253sh (3.3 × $10^6$) and 225 (4.1 × $10^6$); $\nu_{max}$ ($CHCl_3$)/$cm^{-1}$ 3020, 1802, 1619, 1522, 1340, 1161, 1128, 1092, 1073 and 1035; $\delta_H$(400 MHz, $CDCl_3$, $SiMe_4$) 1.46 (3 H, d, *J* 6.69, Me), 2.50 (1 H, s, OH), 4.62 (1 H, m, *CH*OH), 7.56 (1 H, d, *J* 3.94, NCH), 7.91 (1 H, d, *J* 9.52, 6-H-Ph), 8.32 (1 H, dd, *J* 9.60 and 2.54, 5-H-Ph), 9.12 (1 H, d, *J* 2.58, 3-H-Ph) and 11.08 (1 H, s, NH); $\delta_C$(400 MHz, $CDCl_3$, $SiMe_4$) 20.9 (Me), 66.9 (CHOH), 116.3 (Ph), 123.3 (Ph), 130.0 (Ph) and 152.5 (CN); *m/z* (CI, $NH_3$) 272 ($M+NH_4^+$, 39%), 255 ($M+H^+$, 38), 254 ($M^+$, 17) and 237 (39); $R_f$ (TLC; ethyl acetate-light petroleum, 1:1, v/v) 0.32. (*R*)-Lactaldehyde-2,4-dinitrophenylhydrazone was obtained in an identical manner from (*R*)-

lactaldehyde. The $R_f$ (TLC), $^1H$ NMR and mass spectra were indistinguishable from those obtained with the racemic material.

**2.4.10** *Pyruvamide.*— Pyruvamide was synthesised essentially by the method of Anker[250]. A solution of pyruvonitrile (1 g, 14.5 mmol) in anhydrous ether (30 $cm^3$) was saturated with dry HCl under an atmosphere of dry nitrogen. Distilled water (261 $mm^3$, 14.5 mmol) was added and the dry HCl stream was continued for a further 0.5 h. The solvent was removed under reduced pressure and the resulting white solid was recrystallised from ethyl acetate to give pyruvamide (312 mg, 25%), m.p. 110-124 °C (from EtOAc) (lit.,[250] 127 °C); $\delta_H$(220 MHz, $CDCl_3$, $SiMe_4$) 2.5 (3 H, s, $CH_3$); *m/z* (CI, $NH_3$) 105 ($M+NH_4^+$, 100%). The $^1H$ NMR spectrum indicated the presence of water of crystallisation (1 mol $H_2O$ per 4 mol pyruvamide) which presumably depressed the m.p.

**2.4.11** *Hexandial.*— Sodium periodate (0.6 g, 4.3 mmol) was added to a solution of *trans*-1,2-cyclohexandiol (0.5 g, 4.3 mmol) in distilled water (20 $cm^3$). The mixture was stirred for 0.5 h at ambient temperature. A second portion of sodium periodate (0.6 g, 4.3 mmol) was added. After stirring for a further 1.5 h, the mixture was extracted with diethyl ether (5 × 40 $cm^3$). The combined organic extracts were dried ($MgSO_4$) and the solvent was removed under reduced pressure to give hexandial as a colourless oil (353 mg, 72%) (lit.,[251]), $\delta_H$(220 MHz, $CDCl_3$, $SiMe_4$) 1.68 (4 H, m, $CH_2(CH_2)_2CH_2$), 2.52 (4 H, m, 2 × $CH_2CHO$) and 9.85 (2 H, t, *J* 3, 2 × CHO); *m/z* (CI, $NH_3$) 132 ($M+NH_4^+$, 19%), 115 ($M+H^+$, 6), 97 (12), 79 (8), 71 (3) and 70 (11).

**2.4.12** *Characterisation of Biological Acyloins and Miscellaneous Substrates and Products.*— Acyloins produced by ZMPDC were characterised by $^1H$ NMR and GC-mass spectrospcopy either directly from reaction mixtures or from solvent extracts without further purification. The data were consistent with

those obtained with the authentic materials that were prepared by Kren *et al.*[227] and Miyagoshi *et al.*[22617] Additional characterisation of lactaldehyde is described above. The concentration of substrates and products was determined by $^1H$ NMR (220 MHz, $H_2O$, DSS) using the DSS ($\delta_H$ 0.00) and citrate [$\delta_H$ 2.62 (4 H, ddd, *J* 15.4, 15.4 and 15.4, 2 × $CH_2$)] resonances as internal standards. To monitor the concentration of substrates and products with proton resonances which would overlap with those of glycerol [$\delta_H$ 3.55-3.88 (5 H, m)], freshly prepared recombinant ZMPDC was used to which no glycerol was added.

**2.4.12.1** *Aliphatic acetylcarbinols*. (*a*) Acetoin (3-hydroxy-2-butanone), $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 1.39 (3 H, d, *J* 7.11, $CH_3$CH(OH)), 2.20 (3 H, s, $CH_3CO$) and 4.24 (1 H, d, *J* 7.11, CH(OH)); $\delta_H$(220 MHz, $H_2O$, DSS) 1.37 (3 H, d, *J* 7.5, $CH_3$CH(OH)) and 2.24 (3 H, s, $CH_3CO$); *m/z* (EI) 88 ($M^+$, 4%), 73 (2), 59 (1), 57 (1), 45 (100) and 43 (96).

(*b*) Lactaldehyde (hydroxypropanal), $\delta_H$(220 MHz, $H_2O$, DSS) 1.17 (3 H, d, *J* 6.7, $CH_3$(hydrate)) and 3.69 (1 H, m, CH(OH)(hydrate)).

(*c*) 2-Hydroxy-3-pentanone, $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 1.12 (3 H, t, *J* 7.17, $CH_3CH_2$), 1.38 (3 H, d, *J* 7.03, $CH_3$CH(OH)), 2.53 (2 H, m, $CH_2$) and 4.25 (1 H, d, *J* 7.15, CH(OH)); *m/z* (EI) 102 ($M^+$, 1%), 59 (12), 57 (37), 45 (100) and 43 (69).

(*d*) 3-Hydroxy-2-pentanone, $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 0.94 (3 H, t, *J* 7.40, $CH_3CH_2$), 1.62 (2 H, m, $CH_2$), 2.19 (3 H, s, $CH_3CO$) and 4.17 (1 H, dd, *J* 4.01 and 6.86, CH(OH)); *m/z* (EI) 102 ($M^+$, 1%), 59 (71), 57 (11), 45 (22) and 43 (100).

**2.4.12.2** *Aromatic acetylcarbinols*. (*a*) PAC, $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 2.08 (3 H, s, $CH_3CO$), 5.09 (1 H, s, CH(OH)) and 7.3-7.4 (5 H, m, Ph); *m/z* (EI) 150 ($M^+$, 2%), 107 (79), 105 (29), 79 (100), 77 (77), 51 (31) and 43 (25).

(*b*) *o*-Fluoro-PAC, $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 2.13 (3 H, s, $CH_3CO$), 5.40 (1 H, s, CH(OH)) and 7.2-7.4 (4 H, m, Ph); *m/z* (EI) 168 ($M^+$, 1%), 166 (1), 125 (100), 123 (57), 97 (57), 95 (27), 77 (32), 75 (16), 51 (19) and 43 (41).

(*c*) *m*-Fluoro-PAC, $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 2.10 (3 H, s, $CH_3CO$), 5.08 (1 H, s, C*H*(OH)) and 7.0-7.4 (4 H, m, Ph); *m/z* (EI) 168 ($M^+$, 4%), 166 (6), 125 (100), 123 (92), 97 (98), 95 (66), 77 (28), 75 (25), 51 (11) and 43 (44).

(*d*) *p*-Fluoro-PAC, $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 2.08 (3 H, s, $CH_3CO$), 5.08 (1 H, s, C*H*(OH)) and 7.1-7.4 (4 H, m, Ph); *m/z* (EI) 168 ($M^+$, 2%), 166 (2), 125 (100), 123 (88), 97 (47), 95 (42), 77 (11), 75 (12), 51 (3) and 43 (9).

(*e*) *o*-Chloro-PAC, $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 2.13 (3 H, s, $CH_3CO$), 5.58 (1 H, s, C*H*(OH)) and 7.3-7.6 (4 H, m, Ph); *m/z* (EI) 184 ($M^+$, trace %), 143 (15), 141 (50), 139 (12), 115 (4), 113 (15), 111 (9), 77 (100), 75 (15), 51 (37) and 43 (41).

(*f*) *m*-Chloro-PAC, $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 2.10 (3 H, s, $CH_3CO$), 5.06 (1 H, s, C*H*(OH)) and 7.2-7.6 (4 H, m, Ph); *m/z* (EI) 186 (1%), 184 ($M^+$, 3), 182 (1), 143 (17), 141 (60), 139 (22), 115 (8), 113 (31), 111 (17), 77 (100), 75 (20), 51 (23) and 43 (49).

(*g*) *p*-Chloro-PAC, $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 2.08 (3 H, s, $CH_3CO$), 5.06 (1 H, s, C*H*(OH)) and 7.3-7.6 (4 H, m, Ph); *m/z* (EI) 186 (1%), 184 ($M^+$, 2), 182 (1), 143 (28), 141 (95), 139 (44), 115 (6), 113 (24), 111 (23), 77 (100), 75 (22), 51 (23) and 43 (36).

**2.4.12.3** ***Heterocyclic acetylcarbinols.*** 1-Hydroxy-1-(2-furyl)-2-propanone, $\delta_H$(250 MHz, $CDCl_3$, $SiMe_4$) 2.16 (3 H, s, $CH_3CO$) and 5.06 (1 H, s, C*H*(OH)). The heterocyclic acetylcarbinols were not obtained in sufficient yields for further characterisation. They were only identified by GC.

**2.4.12.4** ***Decarboxylation products.*** The data were consistent with those obtained with the commercial authentic materials.

(*a*) Acetaldehyde (ethanal), $\delta_H$(220 MHz, $H_2O$, DSS) 1.34 (3 H, d, *J* 5.2, $CH_3$(hydrate, 50%)), 2.25 (3 H, d, *J* 3.0, $CH_3$) and 9.81 (1 H, q, *J* 3.0, CHO).

(*b*) Glycolaldehyde (hydroxyethanal), $\delta_H$(220 MHz, $H_2O$, DSS) 3.58 (1 H, d, *J* 5.3, CHO(hydrate, 100%)).

(*c*) Acetate (ethanoate), $\delta_H$(220 MHz, $H_2O$, DSS) 1.95 (3 H, s, $CH_3$).

**2.4.12.5** *$^1H$ NMR resonances of substrates used to monitor concentrations.*

(*a*) Pyruvate (2-oxopropanoate) $\delta_H$(220 MHz, $H_2O$, DSS) 1.50 (3 H, s, $CH_3$(hydrate, 8%)) and 2.39 (3 H, s, $CH_3$).

(*b*) 3-Hydroxypyruvate (3-hydroxy-2-oxopropanoate) $\delta_H$(220 MHz, $H_2O$, DSS) 3.72 (2 H, s, $CH_2$(hydrate, 50%)).

(*c*) DL-Serine $\delta_H$(220 MHz, $H_2O$, DSS) 3.88 (1 H, m, CH) and 3.99 (2 H, m, $CH_2$).

(*d*) Glycine $\delta_H$(220 MHz, $H_2O$, DSS) 3.60 (2 H, s, $CH_2$).

(*e*) 3-Bromopyruvate (3-bromo-2-oxopropanoate) $\delta_H$(220 MHz, $H_2O$, DSS) 3.70 (2 H, s, $CH_2$).

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# 3 The Effects of Surfactants on Lipase-Catalysed Hydrolysis of Esters

## 3.1 Introduction

**3.1.1** *The Problem of the Low Water Solubility of Substrates for Biotransformations* — Several strategies have been developed for overcoming difficulties in the biotransformation of water insoluble compounds. To date, the use of organic solvents has proved most useful, particularly with hydrolytic biocatalysts.[1-4] A water-miscible organic solvent can directly increase the solubility of a water insoluble substrate. This strategy is hampered by the fact that enzymes are prone to denaturation by organic solvents[4,5] and that their enantioselectivity can be reduced.[6,7] This effect is dependent on the the nature of the organic solvent and the enzyme. In general, enzymes are most stable to denaturation in hydrophilic organic solvent/aqueous mixtures because hydrophobic organic solvents tend to disrupt the enzyme structure by exposing the enzyme's hydrophobic core.[5] For each enzyme-organic solvent combination, there is a critical threshold concentration of organic solvent at which the enzyme will be denatured,[5,8] thus limiting the effectiveness of this approach. This problem is avoided somewhat, however, when a water-immiscible organic solvent is used. The use of a very hydrophobic, water insoluble organic solvent minimises the contact between it and the enzyme in the aqueous phase.[4,9] The water-immiscible organic solvent can provide a pool of substrate which partitions into the aqueous phase containing the biocatalyst.[10] A drawback of this technique is the possibility that high interfacial energy and low interfacial tension can denature enzymes.[4,10]

In certain cases, it is possible to conduct a biotransformation in a nearly anhydrous organic solvent.[1,2] This strategy is also limited by the nature of

the organic solvent and the concentration of water. Enzymes tend to be most stable to denaturation in hydrophobic organic solvents in nearly anhydrous systems, provided that there is sufficient water present to maintain the essential hydration shell around the enzyme molecules.[1,4] Hydrophilic organic solvents are more able to strip the hydration shell water molecules from the enzyme surface, resulting in enzyme denaturation. Conducting a biotransformation in a nearly anhydrous organic solvent will result in the reversal of an hydrolysis reaction, by shifting the equilibrium position.[1] This reversal of the direction of a reaction can be desirable, but is beyond the scope of the present investigation.

Very little attention has been given to overcoming the problem of low substrate solubility in essentially aqueous environments. Perhaps the most promising strategy is the use of surfactants to disperse water insoluble substrates. Indeed, there are many examples of biosurfactants being secreted by microorganisms to aid the assimilation of hydrocarbons.[11] In order to explain and exploit this strategy, the properties of surfactants need to be understood.

**3.1.2** ***The Properties of Surfactants and Emulsions.***— Surfactant molecules are amphiphilic surface active agents possessing both a hydrophilic "head" and a hydrophobic "tail" (Figure 3.3) which orient themselves accordingly at an interface, such as an oil-water interface.[12] The head region can be either cationic, anionic or nonionic. Some surfactants have distinct head and tail regions, such as phospholipids, while others have less distinct regions, such as the bile salts,[13] which are polycyclic organic compounds with hydrophobic and hydrophilic surfaces. Thus, surfactants can be structurally classified (Table 3.1, Figures 3.1 and 3.2).

**Table 3.1** The characteristics of surfactants[a]

| Surfactant[b] ($pK_a$) | CMC[c] mmol $dm^{-3}$ | N[d] | HLB[e] | Supplier |
|---|---|---|---|---|
| **NONIONIC** | | | | |
| **Alkyl(thio)glucosides and maltosides** | | | | |
| Octylglucoside | 23.2 | 84 | N.f.[f] | Boehringer |
| Octylthioglucoside | 4 | N.f.[f] | N.f.[f] | Sigma |
| Dodecylglucoside | 0.13 | N.f.[f] | N.f.[f] | Boehringer |
| Dodecylmaltoside | 0.18 | 98 | N.f.[f] | Boehringer |
| **Glucamides** | | | | |
| MEGA-8 | 60 | N.f.[f] | N.f.[f] | Boehringer |
| MEGA-10 | 6.2 | N.f.[f] | N.f.[f] | Boehringer |
| **Acylsorbitans (polyethyleneglycolether)** | | | | |
| Tween 80 | 0.012 | 58 | 15 | Sigma and BDH |
| Span 80 | N.f.[f] | N.f.[f] | 4.3 | Sigma |
| **Aromatic polyethyleneglycolethers** | | | | |
| Triton X-100 | 0.2 | 100-155 | 13.5 | Boehringer |
| Triton X-1148 | 0.21 | N.f.[f] | 12.4 | Boehringer |
| **Alkylpolyethyleneglycolethers** | | | | |
| C8E1 | 4.9 | N.f.[f] | 5.2 | Fluka |
| C18E1 | N.f.[f] | N.f.[f] | 2.9 | Fluka |
| C*iso*13E8 | 0.13 | N.f.[f] | 12.8 | Boehringer |
| Thesit (C12E9) | 0.09 | N.f.[f] | 13.6 | Boehringer |
| C12E8 | 0.07 | 120-127 | 13.1 | Fluka |
| Brij 35 (C12E23) | 0.05-0.1 | 40 | 16.9 | Fluka |
| C18E8 | N.f.[f] | N.f.[f] | 11.4 | Fluka |

**Table 3.1** The characteristics of surfactants[a] continued

| Surfactant[b] ($pK_a$) | CMC[c] mmol $dm^{-3}$ | N[d] | HLB[e] | Supplier |
|---|---|---|---|---|
| **Big CHAP** | | | | |
| Big CHAP | 3.4 | 10 | N.f.[f] | Sigma |
| **Polyvinylalcohols** | | | | |
| Rhodoviol 30/5 | N.a.[h] | N.a.[h] | N.a.[h] | Rhone Poulenc |
| Rhodoviol 25/140 | N.a.[h] | N.a.[h] | N.a.[h] | Rhone Poulenc |
| Rhodoviol 4/20 | N.a.[h] | N.a.[h] | N.a.[h] | Rhone Poulenc |
| Rhodoviol 4/125 | N.a.[h] | N.a.[h] | N.a.[h] | Rhone Poulenc |
| PVA 117 | N.a.[h] | N.a.[h] | N.a.[h] | Kurare |
| PVA 205 | N.a.[h] | N.a.[h] | N.a.[h] | Kurare |
| **CATIONIC** | | | | |
| **Quaternary amines** | | | | |
| CTAB | 1 | 170 | N.f.[f] | Sigma |
| Cetylpyridinium.Cl | 0.9 | N.f.[f] | N.f.[f] | Sigma |
| **Alkyldimethylamine oxide** | | | | |
| LDAO (7.0) | 0.14 | 76 | N.f.[f] | Calbiochem |
| **ANIONIC** | | | | |
| **Alkylsulfates** | | | | |
| AOT | N.f.[f] | N.f.[f] | N.f.[f] | Sigma |
| SDS | 1-2 | 101 | 40 | Fluka |
| **Polysaccharide** | | | | |
| Acacia | N.a.[h] | N.a.[h] | N.a.[h] | Aldrich |

**Table 3.1** The characteristics of surfactants[a] continued

| Surfactant[b] ($pK_a$) | CMC[c] mmol $dm^{-3}$ | N[d] | HLB[e] | Supplier |
|---|---|---|---|---|
| **Bile salts** | | | | |
| Cholic acid | N.f.[f] | N.f.[f] | N.f.[f] | H & W |
| Na Cholate (5.2) | (3-10)[i] | (4.8)[i] | 18 | Sigma |
| Na Glycocholate (3.8) | 1-14 | 1.6 | N.f.[f] | Sigma |
| Na Taurocholate (1.9) | 3-6 | 4-5 | N.f.[f] | Sigma |
| Na Deoxycholate (6.2) | (1.4)[i] | (5-19)[i] | 16 | Sigma |
| Na Glycodeoxycholate (4.8) | 1.1 | 19.4 | N.f.[f] | Sigma |
| Na Taurodeoxycholate (1.9) | 1-2 | 12-33 | N.f.[f] | Sigma |
| **ZWITTERIONIC (Sulfo)betaines** | | | | |
| Empigen BB (6.0) | N.f.[f] | N.f.[f] | N.f.[f] | Calbiochem |
| Dodecylsulfobetaine | 3.6 | 55 | N.f.[f] | Boehringer |
| **CHAPS Series** | | | | |
| CHAPS | 7.4 | 4-14 | N.f.[f] | Boehringer |
| CHAPSO | 8 | N.f.[f] | N.f.[f] | Boehringer |

[a]Lit.[12-15] and suppliers. When available, cited data are those determined at pH 7.0, 20 - 25 °C in and the presence of sodium ions (0.1 mol $dm^{-3}$).

[b]Surfactant abbreviations are given in Figure 3.1.

[c]Critical micelle concentration.

[d]Aggregation number. [e]Hydrophile-lipophile balance number. [f]Not found.

[g]Cloud point for Triton X-114 is 22 °C. The cloud point of all other surfactants is greater than 30 °C.

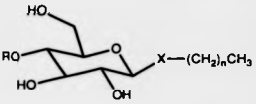
[h]Not applicable.

[i]Appropriate for pH values greater then 8.

**Figure 3.1** Surfactant structures

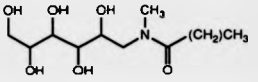
## NONIONIC SURFACTANTS

### ALKYL(THIO)GLUCOSIDES AND MALTOSIDES



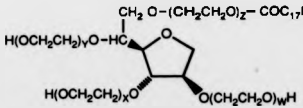

n = 7, X = O, R = H: Octylglucoside (1-*O*-*n*-octyl-β-D-glucopyranoside)
n = 7, X = S, R = H: Octylthioglucoside (1-*O*-*n*-octyl-β-D-thioglucopyranoside)
n = 9, X = O, R = H: Dodecylglucoside (1-*O*-*n*-dodecyl-β-D-glucopyranoside)
n = 9, X = O, R = glucosyl: Dodecylmaltoside
[1-*O*-*n*-dodecyl-β-D-glucopyranosyl-(1→4)-α-D-glucopyranoside]

### GLUCAMIDES

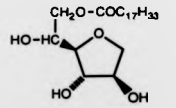



n = 6: MEGA-8 [*N*-(D-gluco-2,3,4,5,6-pentahydroxyhexyl)-*N*-methyloctanamide]
n = 8: MEGA-10 [*N*-(D-gluco-2,3,4,5,6-pentahydroxyhexyl)-*N*-methyldecanamide]

### ACYLSORBITANS (POLYETHYLENEGLYCOLETHER)



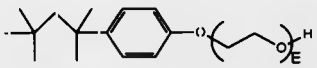

W + X + Y + Z = 20: Tween 80
[sorbitanpoly(ethyleneglycol-ether)$_{20}$ monooleate]




Span 80
(sorbitan monooleate)

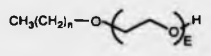
**Figure 3.1** Surfactant structures continued

**AROMATIC POLYETHYLENEGLYCOLETHERS**



E = 10, Triton X-100 [octylphenolpoly(ethyleneglycolether)$_{10}$]
E = 7, Triton X-114 [octylphenolpoly(ethyleneglycolether)$_7$]

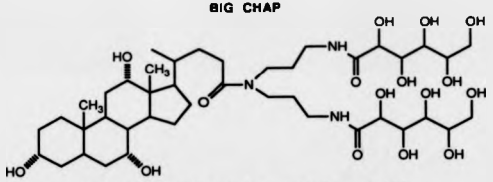
**ALKYLPOLYETHYLENEGLYCOLETHERS**



C = n + 1
E = Number of ethyleneglycolether units

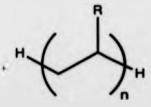
C8E1
C18E1
C$_i$so13E8
Thesit (C12E9)
C12E8
Brij 35 (C12E23)
C18E8

**BIG CHAP**



Big CHAP [*N*,*N*'-di(gluconyl)-cholamidodipropylamide]

**POLYVINYLALCOHOLS (PVAs)**



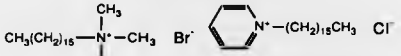
R = OH, OAc

Rhodoviol 30/5
Rhodoviol 25/140
Rhodoviol 4/120
Rhodoviol 4/125
PVA 117
PVA 205

**Figure 3.1** Surfactant structures continued

## CATIONIC SURFACTANTS

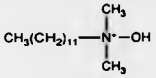
### QUATERNARY AMINES



CTAB (*N*-cetyl*N*,*N*,*N*-trimethylammonium bromide)

Cetylpyridinium chloride

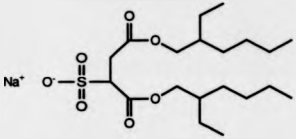
### ALKYLDIMETHYLAMINE OXIDE



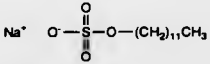
LDAO (*N*-dodecyl*N*,*N*-dimethylamine oxide)

## ANIONIC SURFACTANTS

### ALKYLSULFATES



AOT [Aerosol OT, sodium bis(2-ethylhexyl)sulfosuccinate]



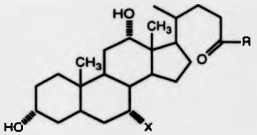
SDS (sodium dodecylsulfate)

### POLYSACCHARIDE

Acacia (gum arabic); acidic polysaccharide; mostly arabinose, galactose, rhamnose and glucuronate; 240,000 - 580,000 molecular weight

**Figure 3.1** Surfactant structures continued

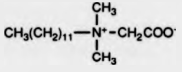
### BILE SALTS



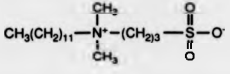
X = OH, R = $O^-Na^+$; Sodium cholate
X = OH, R = $NHCH_2CO_2^-Na^+$; Sodium glycocholate
X = OH, R = $NHCH_2CH_2SO_3^-Na^+$; Sodium taurocholate
X = H, R = $O^-Na^+$; Sodium deoxycholate
X = H, R = $NHCH_2CO_2^-Na^+$; Sodium glycodeoxycholate
X = H, R = $NHCH_2CH_2SO_3^-Na^+$; Sodium taurodeoxycholate

## ZWITTERIONIC SURFACTANTS

### (SULFO)BETAINES

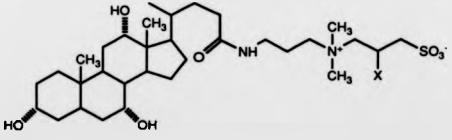


Empigen BB (dodecyl betaine, *N*-dodecyl-*N*,*N*-dimethylglycine)



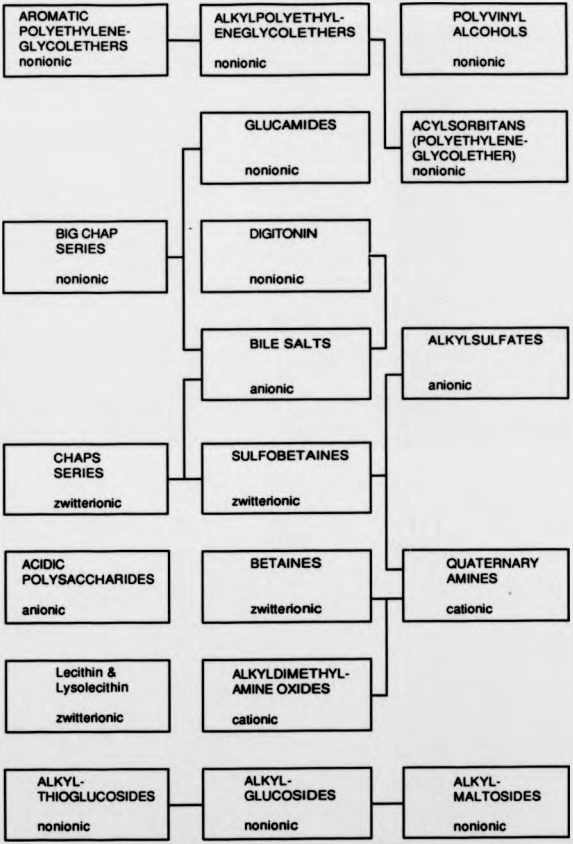
Dodecylsulfobetaine (*N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate)

### CHAPS SERIES



X = H; CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate)
X = OH; CHAPSO (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane-sulfonate)

**Figure 3.2** The structural relationships of common surfactants



Most surfactants exhibit lyotropic mesomorphism, which is the ability to form organised aggregates such as spherical, cubic and lamellar liquid crystals.[16] However, virtually all surfactants are able to form monolayers and micelles.[17] At low concentrations, surfactants dissolve in water as monomers.[12] At higher concentrations, they aggregate to form micelles with hydrophobic cores and with the head groups exposed to the solvent water molecules (Figure 3.3).[12,18] The threshold concentration at which this occurs is called the critical micelle concentration (CMC; Table 3.1). Stable micelles of a given surfactant tend to contain a specific number of surfactant molecules, called the aggregation number (N; Table 3.1). Thus, if the total surfactant concentration, CMC and N are known, the micelle concentration can be calculated. The Krafft point is the melting point of the hydrocarbon chain in the surfactant micelles.[13] Most surfactants are quite insoluble and ineffective below the Krafft point. In addition, above a certain temperature, called the cloud point, the micelles can aggregate to form large insoluble clusters.[12]

**Figure 3.3** Schematic illustration of surfactant molecules and micelles

Surfactants can also be classified by the overall hydrophilicity of the surfactant molecules. An empirical measure, called the hydrophile-lipophile balance (HLB; Table 3.1),[13,14,16,19,20] has been developed to quantify this property. The larger the HLB the more hydrophilic the surfactant. This can be determined experimentally by either the partition coefficient between water and an organic solvent or from the cloud point. The HLB of a number of surfactant substructures has been determined. These can be used to estimate the HLB of a surfactant consisting of a combination of these substructures. The HLB of mixtures of surfactants can also be estimated by the algebraic additivity of the relative contributions from each surfactant.

Surfactants can be removed from aqueous and organic solvent-containing systems by phase partition, hydrophobic absorption chromatography, ion exchange chromatography, affinity chromatography, gel permeation chromatography, dialysis, ultrafiltration, precipitation and many other techniques.[12]

Surfactants are able to disperse water insoluble compounds by a variety of mechanisms. An extreme example[21] is the solubilisation of several moles of the water insoluble condensation products of diethanolamine with the fatty acids of coconut oil by one mole of the water-soluble fatty acids. This phenomenon has been explained by the formation of micelles containing both amphiphilic compounds, called mixed micelles or comicelles.[22] The solubilisation ability is a function of the co-solubility of the compound and the surfactant, and therefore a function of the surfactant HLB.[23] In addition, water insoluble compounds can dissolve in the interior hydrophobic core of micelles, forming microemulsions.[24-28] These swollen micelles form spontaneously and are thermodynamically stable. Since the swollen micelles are typically much smaller than 0.1 μm in diameter, microemulsions are optically transparent. Mixtures of two immiscible liquids and a surfactant

can undergo phase transitions, depending on the relative quantities of each component.[24] Phase diagrams can be constructed showing regions of microemulsions, reverse microemulsions (when the hydrophobic liquid is the continuous phase), liquid crystals, two-phase systems and intermediate phases. However, there are considerably fewer examples of oil-in-water than water-in-oil microemulsions.[15,23] On the other hand, there are many examples of oil-in-water emulsions.[15,17,23] Emulsions are formed when two immiscible liquids are subjected to high shear force or sonication.[29] Small droplets of the internal phase are dispersed in the external phase. The droplet size can be reduced by increasing the shear force when the emulsion is prepared. Emulsions are optically opaque because the typical droplet size is between 0.1 μm and 10 μm. Emulsions are not thermodynamically stable because interfacial tension promotes droplet coalescence resulting in the concomitant reduction of the interfacial area and the free energy of the system.

Emulsions break by a series of events.[17,23] The droplets can aggregate without coalescence and this is called flocculation. Creaming, the separation of a phase rich in droplets of the internal phase, results from a density difference between the two phases. This can be facilitated by and result in flocculation. Creaming can be minimised by adjusting the relative densities of the external and internal phases and lowering the droplet size. Coalescence can occur either directly or after such aggregation events, leading to complete phase separation of the internal and external phases. The rate of breakdown of an emulsion can be slowed down by reducing the rate of creaming, increasing the droplet surface charge, sterically reducing the likelihood of coalescence (for example forming a mechanically strong interfacial film), increasing the viscosity of the external phase and reducing the interfacial tension (also known as interfacial pressure) between the two phases. Emulsions can be broken by centrifugation, freezing, distillation,

filtration and the addition of another surfactant to adjust the HLB (see below).

Surfactants can increase the stability of emulsions[17,23] very effectively by reducing the interfacial tension and to a lesser extent by the other mechanisms listed above. For example, acacia (gum arabic) and polyvinyl alcohols (PVA) can reduce interfacial tension, sterically reduce the rate of coalescence, reduce particle size and increase the viscosity of the external phase while phospholipids can reduce interfacial tension, reduce particle size and increase droplet surface charge. Very stable emulsions are rarely obtained with single surfactants. Mixtures of surfactants can, however, stabilise emulsions almost indefinitely. The ability of surfactants to stabilise emulsions is predominantly a function of HLB. Surfactants and surfactant mixtures with an HLB of between 8 and 15 are most able to support stable oil-in-water emulsions. By contrast, values of between 3 and 6 are appropriate for water-in-oil emulsions. It must be stated that HLB is a rough correlation of function and not of precise efficacy.[20] A consequence of this is that, for a given liquid two-phase system, the optimum combination and HLB of surfactants required for successful emulsion stabilisation must be determined with a degree of trial and error.[17,23]

Surfactants may be able suitably to disperse water insoluble substrates for biotransformations. The effects that surfactants have on enzymes, and proteins in general, need to be considered, in order to explore the potential of this strategy fully.

**3.1.3** ***The Application of Surfactants in Biotechnology and the Interaction of Surfactants with Enzymes.***— Biological surfactants, known as lipids, comprise a significant proportion of all cells. Therefore, all enzymes, in principle, are exposed to surfactants *in vivo*. Many proteins interact with,

and indeed require, lipid bilayers for their biological activity.[18] Integral membrane proteins traverse lipid bilayers and therefore require a membrane to maintain their stable biologically active conformation. This requirement may not be obligatory with peripheral membrane proteins which do not traverse the lipid bilayer but possess some hydrophobic lipid binding regions. The requirement of bound lipid has made the purification of integral membrane proteins very difficult. This problem has been overcome by using biological and synthetic surfactants[12,13,15] to displace these proteins from membranes by disrupting the membranes and replacing the physiological lipids with the added surfactant. There are many examples of this technique, utilising a vast range of biologically compatible surfactants. The biological activity of membrane proteins can then be studied *in vitro* with artificial surfactant membranes.[14] An extension of this methodology is the purification of proteins with reverse micelles.[25-28]

There has been great interest in the use of biocatalysts in reverse micelles, microemulsions,[25-28,30-32] liquid membranes[33] and lyotropic liquid crystals.[34] The biocatalyst is located in the aqueous micro-environment of the interior of the reverse micelles. The most frequently used surfactants are AOT and CTAB (Figure 3.1). The continuous hydrophobic external phase is able to dissolve water insoluble substrates which are able to diffuse into the reverse micelles where the biocatalysis is able to occur. In addition, water soluble and surface active substrates can be used in such systems. By adjusting the water content of the system, the thermodynamic equilibrium of an hydrolytic reaction can be controlled. The problems associated with this technology include the difficulty in preparing the systems while retaining the activity of the biocatalyst. As a result, there are few examples of the use of this technique on a preparative scale. A related use of surfactants in biotransformations is the coating of enzymes with a surfactant,[35,36] by co-precipitation followed by lyophilisation. This allows the enzyme to dissolve in

organic solvents, while retaining catalytic activity. There are therefore many examples of the use of biologically compatible surfactants in biotransformations in essentially low water systems. But, as we shall see, there are fewer examples in essentially aqueous environments.

There have been attempts to mimic the benefit of microbial biosurfactants in the assimilation of water insoluble compounds[11] in whole cell biotransformations. To that end, Triton X-100 has been added to yeast-catalysed biotransformations.[37,38] However, control reactions, without surfactant, were not reported. Surfactants increased the rate of *Mycobacterium* sp.-catalysed reactions,[39] but this was attributed to reducing cell aggregation. In one paper it is suggested that the beneficial effect of Tween 80 on the rate of a biotransformation is as a result of its assimilation as a carbon source.[40] The bioconversion of β-ionone by *Aspergillus niger* was found to be completely inhibited by Triton X-100 and sodium dodecylsulfate (SDS).[41] An interesting effect of surfactants on the relative production of malate and fumarate by *Brevibacterium* sp. has been described.[42] This illustrates the effect of surfactants, not on conversion of a supplied substrate, but on the internal cellular metabolism. Finally, the conversion of cycloalkanones by *Pseudomonas* sp. was found to be stimulated by the nonionic surfactants Triton X-405 and Tween 80.[43] By contrast, anionic and cationic surfactants reduced the reaction rate. It was concluded that the beneficial effect of the nonionic surfactants was not attributable to their surfactant properties. One can, somewhat surprisingly, conclude that there are apparently no examples of the beneficial effect of the dispersion of water insoluble compounds by surfactants for whole cell-catalysed biotransformations.

The effect of surfactants on isolated water soluble enzymes has been studied in a little more detail. It appears that specific surfactant-protein interactions

can stimulate catalytic activity in broad range of enzymes. Dehydrogenases were found to be stimulated specifically by bile salts[44] and Tween 80.[45] Rabbit 3-phosphoglycerol dehydrogenase is activated by Brij 35 but is inhibited by Triton X-100 and deoxycholate.[46] A plant polyphenoloxidase was reported to be activated by SDS.[47] Triton X-100 is routinely added to sialyl transferase-catalysed reactions.[48,49] There is a requirement for surfactants, such as SDS, bile salts, lysolecithin and Tweens, for the activity of the β-carotene cleavage enzyme.[50] Pyruvate oxidase is activated by SDS, nonionic and cationic surfactants.[51] SDS is required absolutely for *Xenopus* tyrosinase activity[52] and this enzyme is stable to denaturation at relatively high concentrations of SDS. Triton X-100 stimulates the rate of luciferase catalysis by several orders of magnitude.[53] In this case, there appears to be a specific surfactant-protein interaction which promotes the release of the product from the enzyme-product complex. Finally, nicotinamide adenine dinucleotide: arginine: adenosine diphosphate ribosyltransferase is activated by micelles of lysolecithin, Triton X-100 and CHAPS by dispersing aggregates of the enzyme.[54]

All of the above examples attribute the activation of water soluble enzymes to specific surfactant-protein interactions, and not to the dispersal of the substrate. This explanation is reasonable because extensive study of the binding of surfactants has shown that many proteins have specific regions which can bind monomeric surfactants, resulting in conformational changes.[15,18] Surfactants with a low CMC are generally non-denaturing because the monomer concentration is too low to have a significant effect on proteins.[5] Cooperative binding of surfactants and micelles does not occur to the native state of water soluble proteins. By contrast, membrane proteins bind surfactants and micelles cooperatively without conformational changes.[5]

Surfactant-induced conformational changes of water soluble proteins do not necessarily lead to more active enzymes, as denaturation events can also ensue.[55] For example, dehydrogenases have been selectively denatured by surfactants as a step in a purification procedure.[56] Surfactants can bind to proteins cooperatively, leading to complete denaturation of a globular protein to form a random coil conformation.[5,18] This phenomenon has been exploited in the estimation of protein molecular weights by SDS electrophoresis.[57] In general, anionic surfactants are more able to denature proteins than cationic surfactants.[15,18] The longer the hydrophobic tail, the more denaturing the surfactant.[15] The requirement of a charged head group is illustrated by the fact that nonionic surfactants do not cooperatively bind to, and denature proteins.[5,15,19] Furthermore, nonionic surfactants bind to specific sites on a protein[18] and rarely induce conformational changes.[16] The denaturation of a protein by one surfactant may be reduced, and indeed reversed, by the addition of another surfactant, which binds more tightly to the protein and ties up the denaturant in comicelles.[55,58]

One notable omission in the discussion above is the effect of surfactants on lipolytic enzyme conformation and activity. These water soluble enzymes are a discrete subset of enzymes, in that they catalyse reactions at an interface. Therefore, lipases are ideal enzymes with which to study the effect of surfactants on biotransformations.

**3.1.4** ***Interfacial Activation of Porcine Pancreatic Lipase and the Effect of Surfactants.*** — Lipases, from both mammalian and microbial sources, are the most commonly used enzymes in biotransformations.[3] They possess the same catalytic properties as proteases and esterases except that they function at interfaces.[59,60] The interface may be the oil-water interface of a water insoluble substrate or the solid-aqueous interface of a hydrophobic surface, such as silconised glass beads or polystyrene beads.[59-62] Since lipases are

heterogeneous catalysts, they do not obey normal Michaelis-Menten kinetics.[63] However, the $K_m$ may be expressed in terms of the interfacial area per volume, rather than the substrate concentration. Lipases differ from peripheral membrane proteins in that they do not have a distinct hydrophobic binding region, and therefore, most resemble water soluble proteins.[60] The crystal structures of human pancreatic lipase[64] and *Mucor miehei* lipase[65] have been published recently. It has become clear that the serine protease catalytic triad (serine, histidine and aspartate) is also present in lipases. The catalytic mechanism is shown in Figure 3.4. Thus lipases are capable of ester hydrolysis, esterification and alcoholysis reactions (Figure 3.5).

**Figure 3.4** The mechanism of lipase catalysis

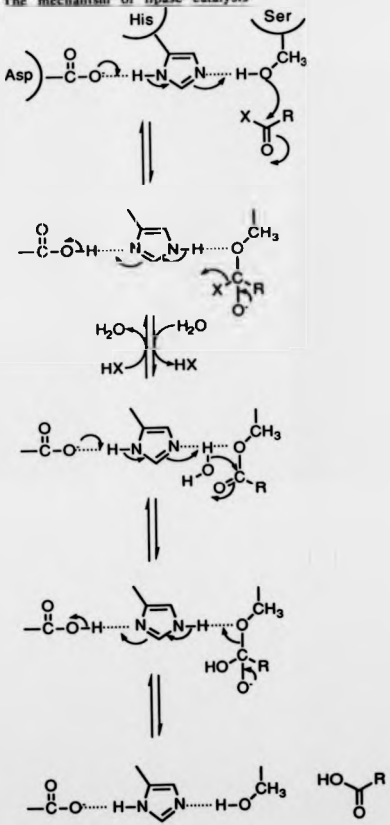
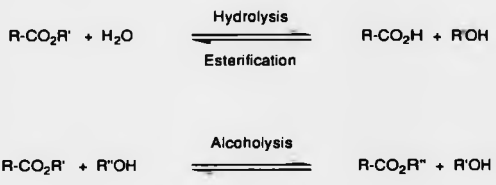


**Figure 3.5** Reactions catalysed by lipases




Although some catalytic activity has been detected with water soluble substrates, lipases are many orders of magnitude more active at interfaces.[59,60] It has been suggested that these water soluble substrates do, in fact, form micellar-like aggregates which permits their hydrolysis by lipases. At this point it worth mentioning that a proposed, second active centre in lipases, capable of hydrolysing water soluble substrates is most probably an artifact.[66,67]

The activation of lipases at interfaces involves conformational changes. This is clearly necessary on examination of the crystal structures.[64,65] The active centre of lipases is buried under a surface polypeptide loop. Significant conformational changes are required for the large, physiological, triglyceride substrate to be able to bind to the active centre. Such conformational changes have been detected.[68] The actual interfacial activation mechanism is poorly understood, but it is, nevertheless, an important property of lipases.

The ability of a lipase to bind to an interface is a function of the surface quality.[59,60] A quantifiable measure of surface quality is the interfacial tension and lipases appear to bind to surfaces within a range of interfacial

tension.[69,70] It is rather more difficult to describe the effect of compounds, other than the substrate, present at the interface.[59,60] The interfacial substrate concentration may be diluted by products, surfactants and proteins. The kinetics of this phenomenon is indistinguishable from classical competitive inhibition. Furthermore, the substrate and surfactant molecules may not be completely miscible, restricting the substrate molecules to isolated pools or domains. Since, at physiological pH, most lipases are negatively charged, cations, such as sodium and calcium ions, can counteract repulsive forces between the lipase and a negatively charged interface.

The most studied lipase is porcine pancreatic lipase (PPL; triacylglycerol acyl hydrolase, EC 3.1.1.3).[59,60] This enzyme is a glycosylated enzyme with a molecular weight of 52 000. Variation in the degree of glycosylation has been observed. This enzyme has six disulfide bridges and two free sulfhydryl groups. The primary structure of PPL[71] is homologous to human[64] and canine[72] pancreatic lipase. The N-terminal domain of human pancreatic lipase is the catalytic domain.[64] The C-terminal domain is present in PPL and other pancreatic lipases,[64] but not in microbial lipases. It may be involved with the interaction of colipase, a cofactor of PPL, which is discussed below.

The physiological role of lipases is the hydrolysis of dietary glycerides.[59,60] This is an important process as only the product fatty acids and glycerol can be absorbed by the intestine. The substrate specificity of PPL is fairly broad as triglycerides, with various fatty acyl chain lengths, and many other esters are hydrolysed. The dietary fat requires emulsification by bile salts to enable the lipase to function efficiently. This is because it has been shown that the rate of lipolysis is dependent on the surface area of substrate. In addition, bile salts tend to protect PPL from interfacial denaturation. Although the rate of hydrolysis by PPL is increased by low concentrations of bile salts, higher concentrations lead to inhibition. (The word inhibition is, and will be, used

rather loosely to mean the reduction of reaction rate.) This effect is due to the bile salts competing with the lipase for interfacial binding sites and the general effects of these detergents on the quality of the interface.

PPL has a non-obligatory cofactor, called colipase.[59,60] This is a heat stable, glycosylated protein containing five disulfide bridges and with a molecular weight of about 10 500. Colipase does not increase the catalytic efficiency of PPL. Its role in lipolysis is to enhance the ability of the lipase to bind to, and orient at, an interface. The exact mechanism is poorly understood, but involves the formation of a lipase-colipase-interface complex. In addition, this complex appears to confer a conformational change in PPL which increases the stability of PPL with respect to interfacial denaturation. Thus, the inhibition of PPL by high concentrations of bile salts is alleviated by the presence of colipase.

There have been many studies on the effect of natural and synthetic surfactants on the rate of hydrolysis of triglycerides. The addition of acacia to lipase assays is routinely practised as substrates, such as triolein, require a surfactant to form stable emulsions.[59,60] Many researchers have reported the stimulation of reaction rates by virtually all types of surfactant.[59,60,68,73-76] However, the stimulatory effect of surfactants is dependent on the source of the lipase, the nature of the substrate and surfactant. All surfactants inhibit lipases at relatively high concentrations; many do so below their CMC.[59,60,73-80] The mechanisms of these effects are similar to those, discussed above, for bile salts, except that some surfactants, such as SDS, are also able to irreversibly denature PPL.[78] For example, colipase alleviates the inhibitory effects of surfactants to some degree, provided the surfactant concentration is not too high.[77-81] The addition of a non-inhibitory surfactant can reduce the inhibition of a lipase by another surfactant, presumably by the formation of comicelles.[82]

Similar observations have been made with non-natural substrates, although no systematic studies have so far been reported. In some cases, there is an apparent requirement of surfactants for lipolytic biotransformations.[83,84] Other reports describe both the stimulatory and inhibitory effects surfactants,[85,86] confirming the dependence of these effects on the nature of the enzyme, substrate and surfactant. Some researchers have added surfactants to biotransformations without reporting control reactions, without surfactant.[87,88] Surfactants could affect the conformation of lipases, either directly or by altering the quality of the interface. Surprisingly, only one paper describes the effect of surfactants on enantioselectivity.[85]

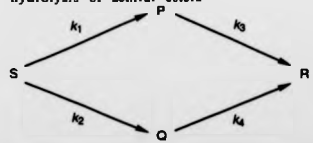
Therefore, it would be of interest to systematically study the effects of surfactants on the rate and enantioselectivity of PPL catalysed biotransformations of unnatural substrates. To that end, the kinetics of enantioselective reactions require consideration.

**3.1.5** *The Kinetics of Enantioselective Reactions.*— Optical purity is normally described in terms of enantiomeric excess (ee).

$$ee\% = \frac{\text{major enantiomer - minor enantiomer}}{\text{major enantiomer + minor enantiomer}} \times 100\ \%$$

The kinetics of the resolution of a achiral substrate by enantiotopos differentiation has been describe by Sih and co-workers.[89]

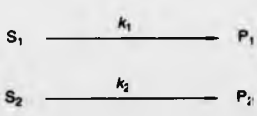
**Figure 3.6** The hydrolysis of achiral esters



Let us consider the achiral substrate S, which is converted to the enantiomeric products P and Q, which can both be converted to the final achiral product, R (Figure 3.6). If $k_2$, $k_3$ and $k_4$ are negligible, the reaction will stop at 50% conversion with a quantitative yield of the optically pure product, P. If $k_3$ and $k_4$ are negligible and $k_1$ is only slightly larger than $k_2$, the reaction will be partially enantioselective, giving P as the major product and Q as the minor product. In this case, the ratio of the products P and Q, and therefore the ee, will remain constant up to a conversion of 50%. If $k_3$ and $k_4$ are small but significant, the ee will also remain essentially constant provided the substrate has not been consumed. When the degree of conversion has exceeded about 50% (in other words, S has been completely converted to products P and Q), the ee may change, depending on the relative values of $k_3$ and $k_4$. If $k_3/k_4$ is equal to $k_1/k_2$ the ee will remain unchanged. If $k_3/k_4$ is greater then $k_1/k_2$, the ee will decrease with conversion and *vice versa*.

The kinetics of the resolution of a racemic substrate by enantiomer differentiation (Figure 3.7) are quite different.

**Figure 3.7** The hydrolysis of chiral esters



It is difficult to compare the enantioselectivity of reactions that have been carried out to different degrees of conversion because the enantiomeric purities of the substrate and the product vary with the degree of conversion. It has been established that the degree of enantioselectivity exhibited by a catalyst can be described by the enantiomer ratio (E).[90]

The E value is a dimensionless constant which is a function of the degree of conversion and the ee of either the substrate or the product:

$$E = \frac{\ln[(1 - c)(1 - ee_s)]}{\ln[(1 - c)(1 + ee_s)]} \qquad E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]}$$

where c is the degree of conversion; $ee_s$ and $ee_p$ are the enantiomeric purity of the substrate and product, respectively.

The degree of conversion may be calculated from the enantiomeric purity of the substrate and product.

$$c = \frac{ee_s}{ee_s + ee_p}$$

The E value is an extremely valuable parameter because it enables the enantioselectivity of catalysts to be compared directly. In addition, E can be used to predict $ee_s$ and $ee_p$ for any given value of c.

**3.1.6** *Aims*.— The aims of this study are to investigate the effects of surfactant on the rate and enantioselectivity of PPL-catalysed biotransformations of water insoluble unnatural substrates. It seems appropriate to use PPL because it is one of the most frequently used biocatalysts, it is active at interfaces and some information regarding the effects of surfactants is available.

### 3.2 Results

The use of either acacia or PVAs to stabilise emulsions of long-chain triacylglycerols in lipase assays is routine.[59,60] The beneficial effect of the use of these surfactants in the PPL-catalysed hydrolysis of olive oil (which is primarily composed of triolein[91]) was confirmed (Table 3.2). The addition of acacia increased the rate of reaction by nearly a factor of 6. PVAs also increased the reaction rate, but the rate enhancement was not as great as with acacia.

**Table 3.2** The effect of surfactants on the rate of PPL-catalysed hydrolysis of olive oil[a]

| Surfactant [concentration/(%w/v)] | Maximum initial linear rate nmol $min^{-1}$ |
|---|---|
| None | 850[b] |
| Acacia (10) | 4970[b] |
| Rhodoviol 4/125 (0.2) | 2370 |
| Rhodoviol 4/125 (2) | 2090 |
| Rhodoviol 25/140 (2) | 2460 |

[a]Crude porcine pancreatic lipase (PPL; 18 mg) was added to a mixture (15 $cm^3$) consisting of an emulsion of purified olive oil (132 $mm^3$) in a solution of NaCl (0.1 mol $dm^{-3}$), $CaCl_2$ (0.02 mol $dm^{-3}$) and surfactant. The mixture was stirred rapidly at 23°C and titrated continuously to pH 8.0 with NaOH (0.2 mol $dm^{-3}$).

[b]Average of duplicate experiments. Error was estimated to be ± 3%.

Suitable substrates were required in order to assess the effect of surfactants on the rate of the PPL-catalysed hydrolysis of water insoluble unnatural substrates. 1-Phenylethylacetate[92-94] (3.1) and 2-phenylpropane-1,3-diol diacetate[95-97] (3.6) have been resolved with biocatalysts. These substrates were screened for PPL-catalysed enantioselective hydrolysis (Table 3.3; Figure 3.8).

Table 3.3 The substrate specificity of PPL[a]

| Substrate | Specific activity nmol $min^{-1}$ mg $protein^{-1}$ | Conversion (%)[b] | ee (%)[b] |
|---|---|---|---|
| Olive oil | 6550 | N.d.[c] | N.d.[c] |
| Tributyrin | 140000 | N.d.[c] | N.d.[c] |
| 1-Phenylethanol acetate (3.1) | 7 | N.d.[c] | N.d.[c] |
| 1-Phenylethanol butanoate (3.2) | 106 | 52 | 63[d] |
| 2-Phenylpropane-1,3-diol diacetate (3.6) | 274 | 50 | 81 |

[a]All reaction mixtures were continuously titrated to pH 8.0. PPL is comprised of 15% protein. See the experimental section for further details.

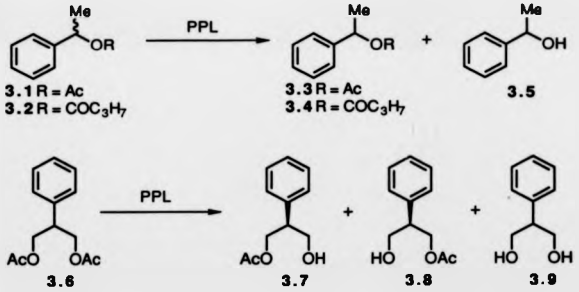
[b]Determined by chiral HPLC.

[c]Not determined.

[d]The remaining substrate had a ee of 70%. The E value was calculated to be 10.

1-Phenylethylacetate (3.1) was an unsuitable substrate because the specific activity of PPL towards this substrate was several orders of magnitude smaller than those for the triacylglycerol substrates. PPL is known to be more active towards butanoate esters than acetate esters.[60] The low specific activity towards 1-phenylethylacetate (3.1) was most probably a consequence of the substrate specificity of PPL rather than any other factors because the corresponding butanoate ester 3.2 was hydrolysed at a much faster rate. The enantiomers of the butanoate 3.2 and their corresponding hydrolysis products (3.5) were resolved by chiral HPLC (Figure 3.9). The absolute configurations of the predominant product and remaining substrate were not determined.

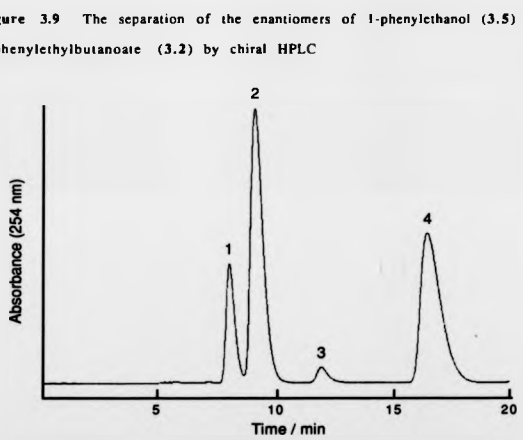
**Figure 3.8** The substrate specificity of PPL



The butanoate ester **3.2** was deemed a suitable substrate to study because it fulfilled three important criteria:

- the substrate was water insoluble
- the activity of PPL towards this substrate was relatively low in the absence of added surfactant, allowing room for improvement
- the enantioselectivity of PPL towards this substrate was moderate in the absence of added surfactant, allowing room for improvement

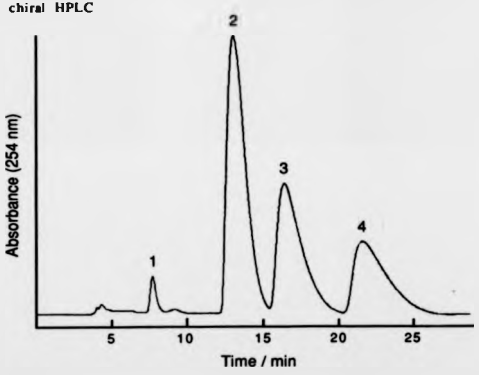
**Figure 3.9** The separation of the enantiomers of 1-phenylethanol (**3.5**) and 1-phenylethylbutanoate (**3.2**) by chiral HPLC




The enantiomers of 1-phenylethanol (**3.5**; peaks 1 and 2) and 1-phenylethylbutanoate (**3.2**; peaks 3 and 4) were separated using a Chiralcel OB HPLC column eluted with 2-propanol-hexane (1:9, v/v) at a flow rate of 0.5 $cm^3$ $min^{-1}$.

2-Phenylpropane-1,3-diol diacetate was also deemed a suitable substrate because it fulfilled all three criteria. There was no need to use the corresponding dibutanoate ester in this case because the activity of PPL towards this substrate was sufficient. The diacetate **3.6** and its corresponding hydrolysis products were resolved by chiral HPLC (Figure 3.10). The predominant product was assumed to be the (*S*)-monoacetate **3.7**, as indicated by Ramos Tombo *et al.*[96,97]

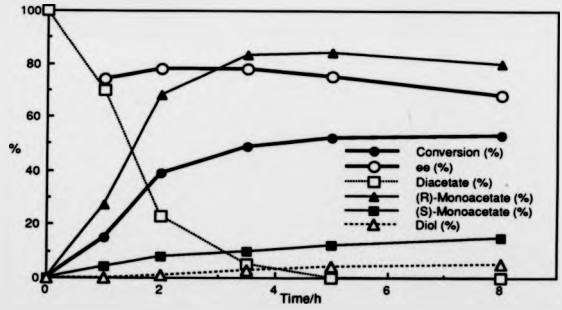
**Figure 3.10** The separation of 2-phenylpropane-1,3-diol (**3.9**), the corresponding diacetate **3.6** and the enantiomers of the monoacetates **3.7** and **3.8** by chiral HPLC




2-Phenylpropane-1,3-diol (**3.9**; peak 1), 2-phenylpropane-1,3-diol diacetate (**3.6**; peak 3), (*S*)-2-phenylpropane-1,3-diol monoacetate (**3.7**; peak 2) and (*R*)-2-phenylpropane-1,3-diol monoacetate (**3.8**; peak 4) were separated using a Chiralcel OB HPLC column eluted with 2-propanol-hexane (3:17, v/v) at a flow rate of 0.75 $cm^3\ min^{-1}$.

2-Phenylpropane-1,3-diol diacetate was chosen as the most appropriate substrate to study because the kinetics of the enzymic resolution of such a achiral compound are more straightforward than those for chiral compounds. This is illustrated in Figure 3.11. The reaction rate approached zero when the diacetate **3.6** was depleted at about 50% conversion, indicating that the monoacetates **3.7** and **3.8** were poor substrates compared with the starting material. The optical purity of the monoacetate product was essentially constant between 0 and 50% conversion, thus allowing the direct comparison of the enantioselectivity of reactions that have proceeded to different degrees of conversion. Similar results have been obtained with the PPL-catalysed hydrolysis of an homologous 2-substituted propane-1,3-diol diacetate.[98]

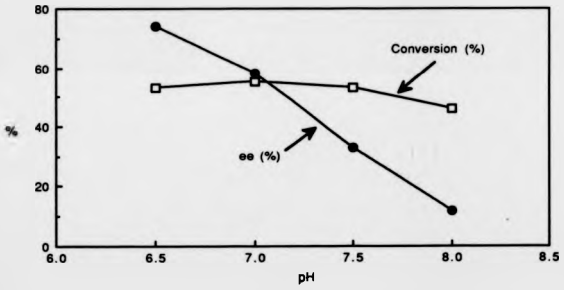
**Figure 3.11** Time course of the PPL-catalysed hydrolysis of 2-phenylpropane-1,3-diol diacetate




Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), 2-phenylpropane-1,3-diol diacetate (10 $mm^3$), NaCl and crude PPL (7.5 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C. Conversion and enantioselectivity were determined by chiral HPLC.

The enantioselectivity of the PPL-catalysed hydrolysis of the diacetate **3.6** was found to be dependent on pH (Figure 3.12). In order to study the effect of added surfactants on this reaction, adequate control of pH was necessary. It is possible that the conformation of PPL may have been affected by pH. Such a conformational change may affect either the enantioselectivity of PPL or the relative rates of hydrolysis of the substrate and intermediate monoacetate products. However, it is also possible that this substrate is hydrolysed by several activities, with different pH optima and enantioselectivities, present in the crude PPL preparation. Evidence for the latter is described below.

**Figure 3.12** The effect of pH on the conversion and enantioselectivity of the PPL-catalysed hydrolysis of 2-phenylpropane-1,3-diol diacetate




Reaction mixtures consisted of sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), 2-phenylpropane-1,3-diol diacetate (10 $mm^3$) and crude PPL (3.75 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h. Conversion and enantioselectivity were determined by chiral HPLC.

The inexpensive, crude PPL enzyme preparation has been frequently used in biotransformations[3] and this material is known to contain a number of distinct hydrolytic activities. Although the genuine triacylglycerol acyl hydrolase (EC 3.1.1.3) activity has been assumed to be the enzyme responsible for the observed esterolytic activity, anomalous results have been obtained whereby enantioselective hydrolyses catalysed by the crude enzyme were not catalysed by the purified enzyme.[99-101]

An apparent example of this phenomenon was reported by Ramos Tombo *et al.*[96,97] who reported the enantioselective hydrolysis of 2-substituted propane-1,3-diol diacetates, including the 2-phenyl derivative (**3.6**). These substrates were hydrolysed by crude PPL but not by a commercial, purified preparation (Sigma). Crude PPL was fractionated by Sephadex G25 chromatography and a pool ("pool 1") containing the higher molecular weight fractions was found to produce material with the best optical purity, particularly when the enzyme activity was immobilised on Eupergit C.

Repetition of some of this work has shown that 2-phenylpropane-1,3-diol diacetate was hydrolysed readily by purified PPL (obtained from both Sigma and Boehringer), to give a product with an optical purity similar to that obtained with the crude enzyme (Table 3.4). It is worth mentioning that acetyl migration, known to occur in 1,3-diols[102], was unlikely to occur at a significant rate in this study. It was also found, in agreement with Ramos Tombo *et al.* that the diester **3.6** was hydrolysed by a cholesterol esterase preparation (EC 3.1.1.13), but with poor enantioselectivity. In addition, Lipase C, an esterolytic enzyme purified from porcine pancreas, was highly active but exhibited no enantioselectivity with this substrate. It is unlikely that this activity is present in the PPL preparations as the enantioselectivity of the crude and purified PPL preparations were similar.

**Table 3.4** Activities of commercial PPL preparations with respect to the hydrolysis of 2-phenylpropane-1,3-diol diacetate[a]

| Enzyme [mg; protein (%)[b]] | Specific activity nmol $min^{-1}$ $mg.protein^{-1}$ | Conversion (%)[c] | ee (%)[c] |
|---|---|---|---|
| Crude PPL[d] (250; 15) | 234 | 50 | 81 |
| Cholesterol esterase[d] (1.2; 74) | 553 | 49 | 52 |
| Purified PPL[d] (0.5; 68) | N.d.[e] | 48 | 76 |
| Purified PPL[d] (1.6; 68) | 705 | 50 | 66 |
| Purified PPL[f] (1.0; 100) | 1850 | 50 | 78 |
| Lipase C[g] (30.5; 42) | 3570 | 51 | 0 |

[a]Reaction mixtures consisted of enzyme, NaCl (0.1 mol $dm^{-3}$; 15 $cm^3$) and 2-phenylpropane-1,3-diol diacetate (60 $mm^3$), and were continuously titrated to pH 7.0.

[b]Determined by the Microprotein Phenol Reagent Method (Sigma Procedure No. 690).

[c]Determined by chiral HPLC.

[d]Obtained from Sigma.

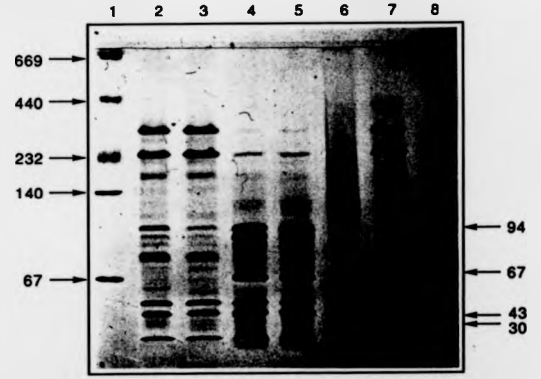
[e]Not determined.

[f]Obtained from Boehringer.

[g]An esterolytic porcine pancreatic enzyme obtained from Enzymatix.

Non-denaturing PAGE (Figures 3.13 and 3.14) revealed a number of proteins in all of the enzyme preparations. The purified PPL from Boehringer was the most homogeneous with, presumably, the true triacylglycerol acyl hydrolase [65 000 - 67 000 molecular weight (Lit. 52 000[71]] being the predominant protein. The same protein was predominant in the purified Sigma preparation but very faint in the crude preparation.

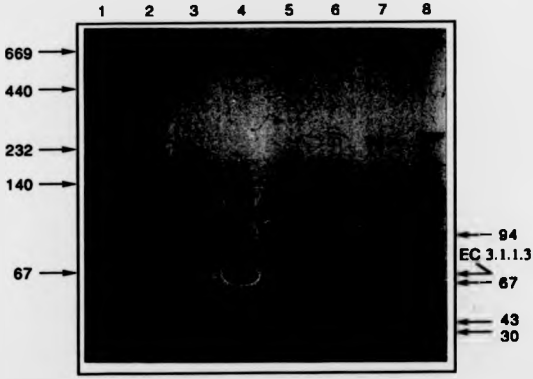
**Figure 3.13** Non-denaturing PAGE of commercial porcine pancreatic enzyme preparations




Electrophoretic separation of high molecular weight markers (channel 1), crude PPL (2), Lipase A (3), purified PPL (Sigma; 4), Lipase B (5), Lipase C (6), cholesterol esterase (7) and low molecular weight markers (8). Molecular weights are shown in thousands.

Lipase A and B (obtained from Enzymatix) were found to be identical to the crude and purified preparations from Sigma. The cholesterol esterase preparation was relatively heterogeneous with many proteins in common with the crude PPL preparation. Finally, Lipase C gave a diffuse band with a molecular weight of between 100 000 and 400 000, which was not present in either of the other preparations. This diffuse band was not a consequence of over-loading, extreme pH or high ionic strength of the sample (data not shown).

**Figure 3.14** Non-denaturing PAGE of the PPL fractions




Electrophoretic separation of high molecular weight markers (channel 1), crude PPL (2), purified PPL (Sigma; 3), purified PPL (Boehringer; 4), fraction 3 (5), fraction 4 (6), fraction 5 (7) and low molecular weight markers (8). Molecular weights are shown in thousands.

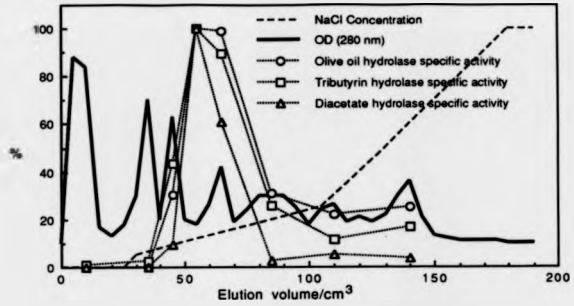
To obtain further evidence on the identity of the active enzymes, crude PPL was fractionated by FPLC using a Mono Q anion exchange column and assayed for hydrolytic activity against olive oil, tributyrin and the diacetate **3.6**. The general activity profiles were common to all substrates, strongly suggesting that the hydrolytic activity towards the diester **3.6** was attributable to the authentic triacylglycerol acyl hydrolase (Table 3.5 and Figure 3.15).

**Table 3.5** Fractionation of PPL by FPLC[a]

| Fraction No. (NaCl concentration/ mol $dm^{-3}$)[a] | Specific activity towards: Olive oil[b] | Tributyrin[b] | 2-Phenylpropane-1,3-diol diacetate[c] |
|---|---|---|---|
| 1 (0) | 0 | 0.7 | 0 |
| 2 (0 - 0.1) | 0 | 2.2 | 0 |
| 3 (0.1 - 0.125) | 2.4 | 36.9 | 19 |
| 4 (0.125 - 0.15) | 7.8 | 84.9 | 217 |
| 5 (0.15 - 0.175) | 7.8 | 75.6 | 132 |
| 6 (0.175 - 0.25) | 2.4 | 21.6 | 6 |
| 7 (0.25 - 0.45) | 1.7 | 9.8 | 11 |
| 8 (0.45 - 0.8) | 2.0 | 14.2 | 8 |

[a]Crude PPL (Sigma) was applied to a Mono Q anion exchange column and eluted with a NaCl gradient (0 - 1 mol $dm^{-3}$) in pH 8.0 Tris buffer (20 mmol $dm^{-3}$).
[b]$\mu$mol $min^{-1}$ mg.protein$^{-1}$.
[c]nmol $min^{-1}$ mg.protein$^{-1}$.

**Figure 3.15** Fractionation of PPL by FPLC




Crude PPL was applied to a Mono Q anion exchange column and eluted with a 0 - 1 mol $dm^{-3}$ NaCl gradient in pH 8.0 Tris buffer (20 mmol $dm^{-3}$). NaCl concentration and OD (280 nm) are expressed as a percentage of a 1 mol $dm^{-3}$ concentration and an OD of 2 respectively. Hydrolase specific activity is expressed as a percentage of the most active fraction (see Table 3.5 for absolute values).

**Table 3.6** Enantioselectivity of FPLC fractions of PPL towards hydrolysis of 2-phenylpropane-1,3-diol diacetate

| Fraction No.[a] | Conversion (%)[b] | ee (%)[b] |
|---|---|---|
| 3 | 30 | 70 |
| 4 | 33 | 87 |
| 5 | 28 | 79 |
| PPL[c] | 32 | 86 |

[a]See Table 3.5.

[b]Determined by chiral HPLC.

[c]An aliquot of the crude material that was applied to the FPLC column.

Further evidence was provided by the finding that the main active fraction (fraction 4) had an elution volume identical with that of the purified enzyme from Boehringer. In addition, this fraction was comprised primarily of a protein of similar molecular weight to that of the Boehringer enzyme (Figure 3.14). The absence of the Lipase C in the other PPL preparations was confirmed by FPLC. Lipase C eluted with a volume of 100 $cm^3$. This protein did not appear in the elution profiles of the other preparations. Moreover, 2-phenylpropane-1,3-diol diacetate hydrolase activity was not detected in the corresponding fraction of crude PPL.

The most active fractions (Table 3.5) exhibited similar enantioselectivities (Table 3.6). The most active fraction gave the highest optical purity which was the same as that obtained with the crude material that was applied to the FPLC column.

On the basis of these results it can be concluded that the hydrolytic activity towards the diacetate **3.6** was that of the true triacylglycerol acyl hydrolase of porcine pancreas.

Ideally, enzyme studies should be conducted with pure enzyme. However, it was most convenient to use the crude preparation as the commercial purified enzyme was very expensive and the purification of the enzyme would have been time consuming.

Forty three surfactants (Fig 3.1), at a standard concentration of 2 mg $cm^{-3}$, were screened for their effect on the rate and stereochemistry of hydrolysis of the diacetate **3.6** (Table 3.7). The same data are presented in order of increasing inhibition in Table 3.8 and plotted graphically in Figure 3.16.

**Table 3.7** The effect of surfactants on the PPL-catalysed hydrolysis of the diacetate **3.6** (organised structurally)[a]

| No.[b] | Surfactant | Conversion (%)[c] | ee (%)[c] |
|---|---|---|---|
| **NONIONIC** | | | |
| **Alkyl(thio)glucosides and maltosides** | | | |
| 14 | Octylglucoside | 49 | 49 |
| 26 | Octylthioglucoside | 32 | 56 |
| 31 | Dodecylglucoside | 27 | 57 |
| 35 | Dodecylmaltoside | 19 | 48 |
| **Glucamides** | | | |
| 17 | MEGA-8 | 48 | 43 |
| 27 | MEGA-10 | 31 | 51 |
| **Acylsorbitans (polyethyleneglycolether)** | | | |
| 23 | Tween 80 (BDH) | 36 | 52 |
| 18 | Tween 80 (Sigma) | 47 | 52 |
| 21 | Span 80 | 41 | 57 |
| **Aromatic polyethyleneglycolethers** | | | |
| 33 | Triton X-100 | 25 | 44 |
| 34 | Triton X-114 | 21 | 53 |

**Table 3.7** The effect of surfactants on the PPL-catalysed hydrolysis of diacetate **3.6** (organised structurally)[a] continued

| No.[b] | Surfactant | Conversion (%)[c] | ee (%)[c] |
|---|---|---|---|
| **Alkylpolyethyleneglycolethers** | | | |
| 13 | C8E1 | 49 | 66 |
| 19 | C18E1 | 46 | 64 |
| 29 | *Ciso*13E8 | 29 | 43 |
| 37 | Thesit (C12E9) | 16 | 37 |
| 38 | C12E8 | 15 | 45 |
| 39 | Brij 35 (C12E23) | 14 | 36 |
| 40 | C18E8 | 10 | 50 |
| **Big CHAP** | | | |
| 32 | Big CHAP | 26 | 35 |
| **Polyvinylalcohols** | | | |
| 7 | Rhodoviol 30/5 | 52 | 57 |
| 8 | Rhodoviol 25/140 | 52 | 56 |
| 9 | Rhodoviol 4/20 | 52 | 60 |
| 11 | Rhodoviol 4/125 | 50 | 56 |
| 3 | PVA 117 | 55 | 58 |
| 5 | PVA 205 | 53 | 62 |

**Table 3.7** The effect of surfactants on the PPL-catalysed hydrolysis of diacetate **3.6** (organised structurally)[a] continued

| No.[b] | Surfactant | Conversion (%)[c] | ee (%)[c] |
|---|---|---|---|
| | **CATIONIC** | | |
| | **Quaternary amines** | | |
| 41 | CTAB | <2 | N.d.[d] |
| 42 | Cetylpyridinium chloride | <2 | N.d.[d] |
| | **Alkyldimethylamine oxide** | | |
| 22 | LDAO | 37 | 37 |
| | **ANIONIC** | | |
| | **Alkylsulfates** | | |
| 30 | AOT | 28 | 57 |
| 43 | SDS | <2 | N.d.[d] |
| | **Polysaccharide** | | |
| 2 | Acacia | 56 | 57 |
| | **Bile salts** | | |
| 10 | Cholic acid | 50 | 61 |
| 6 | Sodium cholate | 53 | 49 |
| 4 | Sodium glycocholate | 54 | 57 |
| 15 | Sodium taurocholate | 48 | 61 |
| 20 | Sodium deoxycholate | 41 | 61 |
| 12 | Sodium glycodeoxycholate | 50 | 47 |
| 25 | Sodium taurodeoxycholate | 34 | 48 |

**Table 3.7** The effect of surfactants on the PPL-catalysed hydrolysis of diacetate **3.6** (organised structurally)[a] continued

| No.[b] | Surfactant | Conversion (%)[c] | ee (%)[c] |
|---|---|---|---|
| | **ZWITTERIONIC (Sulfo)betaines** | | |
| 16 | Empigen BB | 48 | 57 |
| 36 | Dodecylsulfobetaine | 19 | 55 |
| | **CHAPS Series** | | |
| 28 | CHAPS | 31 | 37 |
| 24 | CHAPSO | 36 | 49 |
| | **CONTROL** | | |
| 1 | Without surfactant | 51 | 53 |

[a]Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), 2-phenylpropane-1,3-diol diacetate (10 $mm^3$), surfactant (6 mg) and crude PPL (3.75 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h.

[b]Numbered in order of increasing enzyme inhibition. Control without surfactant is numbered 1.

[c]Determined by chiral HPLC.

[d]Not determined.

**Table 3.8** The effect of surfactants on the PPL-catalysed hydrolysis of the diacetate **3.6**[a]

| No.[b] | Surfactant | Conversion (%)[c] | ee (%)[c] |
|---|---|---|---|
| 1 | Control without surfactant | 51 | 53 |
| 2 | Acacia | 56 | 57 |
| 3 | PVA 117 | 55 | 58 |
| 4 | Sodium glycocholate | 54 | 57 |
| 5 | PVA 205 | 53 | 62 |
| 6 | Sodium cholate | 53 | 49 |
| 7 | Rhodoviol 30/5 | 52 | 57 |
| 8 | Rhodoviol 25/140 | 52 | 56 |
| 9 | Rhodoviol 4/20 | 52 | 60 |
| 10 | Cholic acid | 50 | 61 |
| 11 | Rhodoviol 4/125 | 50 | 56 |
| 12 | Sodium glycodeoxycholate | 50 | 47 |
| 13 | C8E1 | 49 | 66 |
| 14 | Octylglucoside | 49 | 49 |
| 15 | Sodium taurocholate | 48 | 61 |
| 16 | Empigen BB | 48 | 57 |
| 17 | MEGA-8 | 48 | 43 |
| 18 | Tween 80 (Sigma) | 47 | 52 |
| 19 | C18E1 | 46 | 64 |
| 20 | Sodium deoxycholate | 41 | 61 |
| 21 | Span 80 | 41 | 57 |
| 22 | LDAO | 37 | 37 |
| 23 | Tween 80 (BDH) | 36 | 52 |
| 24 | CHAPSO | 36 | 49 |
| 25 | Sodium taurodeoxycholate | 34 | 48 |
| 26 | Octylthioglucoside | 32 | 56 |
| 27 | MEGA-10 | 31 | 51 |
| 28 | CHAPS | 31 | 37 |
| 29 | *Cis*o13E8 | 29 | 43 |

**Table 3.8** The effect of surfactants on the PPL-catalysed hydrolysis of the diacetate **3.6** continued[a]

| No.[b] | Surfactant | Conversion (%)[c] | ee (%)[c] |
|---|---|---|---|
| 30 | AOT | 28 | 57 |
| 31 | Dodecylglucoside | 27 | 57 |
| 32 | Big CHAP | 26 | 35 |
| 33 | Triton X-100 | 25 | 44 |
| 34 | Triton X-114 | 21 | 53 |
| 35 | Dodecylmaltoside | 19 | 48 |
| 36 | Dodecylsulfobetaine | 19 | 55 |
| 37 | Thesit (C12E9) | 16 | 37 |
| 38 | C12E8 | 15 | 45 |
| 39 | Brij 35 (C12E23) | 14 | 36 |
| 40 | C18E8 | 10 | 50 |
| 41 | CTAB | <2 | N.d.[d] |
| 42 | Cetylpyridinium chloride | <2 | N.d.[d] |
| 43 | SDS | <2 | N.d.[d] |

[a]Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), 2-phenylpropane-1,3-diol diacetate (10 $mm^3$), surfactant (6 mg) and crude PPL (3.75 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h.

[b]Numbered in order of increasing enzyme inhibition. Control without surfactant is numbered 1.

[c]Determined by chiral HPLC.

[d]Not determined.

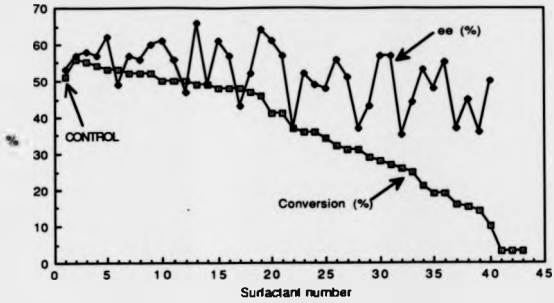
Under the conditions used, the reaction proceeded to about 50% conversion in the absence of surfactant. Since the rate of reaction is known to approach zero after 50% conversion, any rate enhancement as a result of adding surfactant would not be clearly apparent. However, the data shows that the surfactants varied widely in the degree of inhibition of the reaction relative to the system without surfactant. For convenience, the term "inhibition" is used to describe the lowering of conversion in the standard single point determinations.

Some patterns of inhibition can be discerned as illustrated by the data in Table 3.7 in which the results are grouped under the various classes of surfactant. Thus the bile salts, PVAs and acacia gave little inhibition, whereas the quaternary ammonium compounds and alkylsulfates were strongly inhibitory. With the other classes of surfactant, the degree of inhibition was dependent on structure. This is most clearly seen in the alkylpolyethyleneglycolether series, where the degree of inhibition was related to the length of the alkyl and polyethyleneglycolether chains. A similar trend can be seen with the alkyl-(thio)-glucosides and maltosides and with the glucamides, for which the degree of inhibition increased with the length of the alkyl chain. No correlation was found between the degree of inhibition by surfactants and either their CMC or HLB.

With some classes of surfactant, the ee of the monoacetate product was lower than that in the absence of surfactant. This will be considered below.

No single surfactant stabilised an emulsion of the diacetate **3.6**. This is not altogether surprising as the PVAs were used at relatively low concentrations and single surfactants rarely stabilise oil-in-water emulsions.[17,23]

**Figure 3.16** The effect of surfactants on the PPL-catalysed hydrolysis of 2-phenylpropane-1,3-diol diacetate




Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), 2-phenylpropane-1,3-diol diacetate (10 $mm^3$), surfactant (6 mg) and crude PPL (3.75 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h. The surfactants are numbered in order of increasing enzyme inhibition (see Table 3.8). The control without surfactant is numbered 1. Conversion and enantioselectivity were determined by chiral HPLC.

Since PVAs are generally used in lipase assays at relatively high concentrations, the PVA screen reactions were repeated (Figure 3.9) with PVAs at concentrations approaching saturation. Most PVAs did not affect the degree of conversion and the remainder inhibited the reaction. Most of the PVAs stabilised emulsions of the diacetate **3.6**. However, there was no correlation between the stabilisation of an emulsion and the degree of conversion. There was no significant effect on the stereochemistry of the reaction.

**Table 3.9** The effect of PVA stabilised emulsions on the PPL-catalysed hydrolysis of the diacetate **3.6**[a]

| PVA (concentration/mg $cm^{-3}$) | Emulsion[b] | Conversion (%)[c] | ee (%)[c] |
|---|---|---|---|
| None | no | 35 | 68 |
| Rhodoviol 30/5 (0.4) | no | 37 | 73 |
| Rhodoviol 25/140 (16.7) | yes | 24 | 66 |
| Rhodoviol 4/20 (26.7) | yes | 35 | 71 |
| Rhodoviol 4/125 (16.7) | yes | 21 | 70 |
| PVA 117 (0.27) | no | 36 | 72 |
| PVA 205 (16.7) | yes | 21 | 65 |

[a]Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), 2-phenylpropane-1,3-diol diacetate (10 $mm^3$), PVA and crude PPL (3.75 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 2.5 h.

[b]The formation of an emulsion was visually assessed prior to the addition of enzyme and after 2.5 h.

[c]Determined by chiral HPLC.

There have been reports of the benefit of adding a non-inhibitory surfactant to alleviate the inhibition of another surfactant.[82] We have confirmed this using octylglucoside to reduce the inhibition caused by dodecylsulfobetaine and C8E1 (Table 3.10). However, in the conditions used, inhibition was not abolished. Further more, the diacetate **3.6** was not emulsified in any of the systems and a rate enhancement, relative to the control without surfactant, was not observed. There was no significant effect on the stereochemistry of the reaction.

**Table 3.10** The effect of mixed surfactants on the PPL-catalysed hydrolysis of the diacetate **3.6**[a]

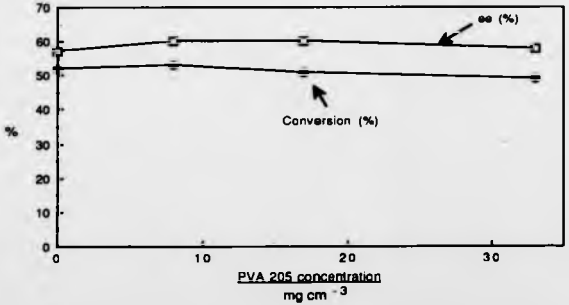
| Surfactant | Conversion (%)[b] | ee (%)[b] |
|---|---|---|
| None | 53 | 56 |
| Octylglucoside | 54 | 58 |
| Dodecylsulfobetaine | 32 | 57 |
| C8E1 | 53 | 58 |
| Octylglucoside and dodecylsulfobetaine | 40 | 56 |
| C8E1 and dodecylsulfobetaine | 43 | 46 |

[a]Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), 2-phenylpropane-1,3-diol diacetate (10 $mm^3$), surfactant (6 mg, each) and crude PPL (3.75 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h.

[b]Determined by chiral HPLC.

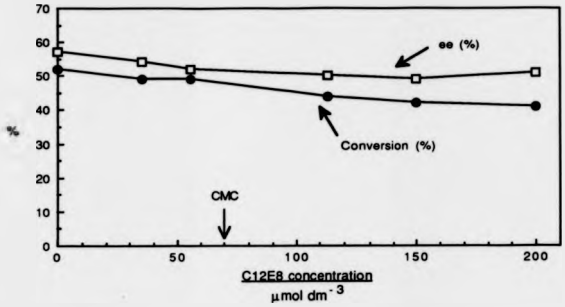
There was no effect of a broad range of PVA 205 concentrations on conversion and enantioselectivity (Figure 3.17), despite the stabilisation of an emulsion of the diacetate **3.6**. Studies were carried out with several surfactants at concentrations spanning the CMC. None of the surfactants stabilised an emulsion of the diacetate **3.6**. There was little or no effect of C12E8 (Figure 3.18) and taurocholate (Figure 3.19) on conversion and enantioselectivity in the concentration range used. However, Tween 80, Brij 35, Triton X-100 and Big CHAP were inhibitory (Figures 3.20, 3.21, 3.22 and 3.23). It appears as though in all cases the reaction was inhibited at concentrations below the CMC and no further significant inhibition occurred above the CMC. In addition, a concomitant reduction in ee was observed with a reduction in conversion with all added surfactants.

**Figure 3.17** The effect of PVA 205 concentration on the PPL-catalysed hydrolysis of the diacetate **3.6**



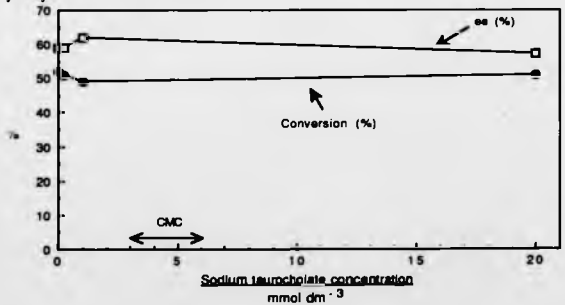

Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), the diacetate **3.6** (10 $mm^3$), surfactant and crude PPL (3.75 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h. Conversion and enantioselectivity were determined by chiral HPLC.

**Figure 3.18** The effect of C12E8 concentration on the PPL-catalysed hydrolysis of the diacetate **3.6**



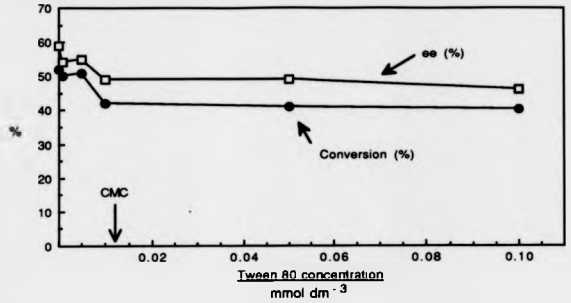

See Figure 3.17.

**Figure 3.19** The effect of taurocholate concentration on the PPL-catalysed hydrolysis of the diacetate **3.6**



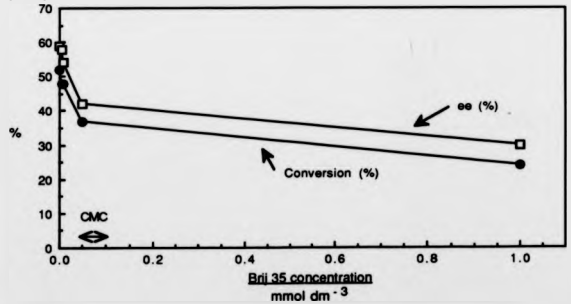

See Figure 3.17.

**Figure 3.20** The effect of Tween 80 concentration on the PPL-catalysed hydrolysis of the diacetate **3.6**



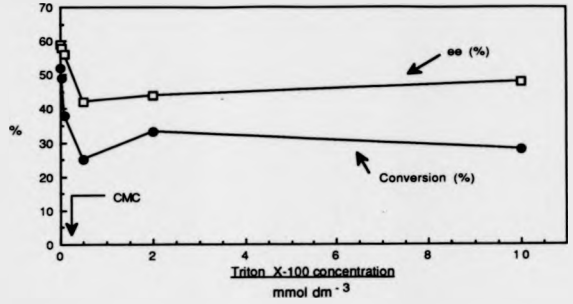

See Figure 3.17.

**Figure 3.21** The effect of Brij 35 concentration on the PPL-catalysed hydrolysis of the diacetate **3.6**



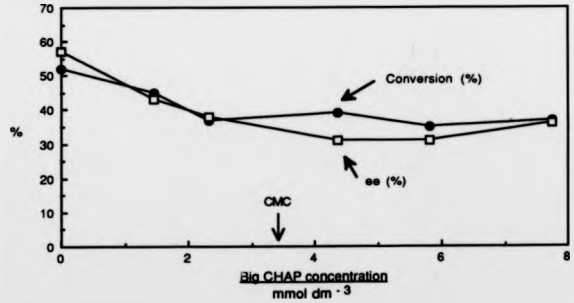

See Figure 3.17.

**Figure 3.22** The effect of Triton X-100 concentration on the PPL-catalysed hydrolysis of the diacetate **3.6**




See Figure 3.17.

**Figure 3.23** The effect of Big CHAP concentration on the PPL-catalysed hydrolysis of the diacetate **3.6**




See Figure 3.17.

As described above, there are few examples of single surfactants stabilising oil-in-water emulsions. Mixtures of surfactants are often very efficient emulsifiers. Empirical observations indicate that the ability of surfactants to stabilise emulsions is a function of HLB. In general, surfactants with an HLB of between[17,23] 8 and 15 are the most effective.

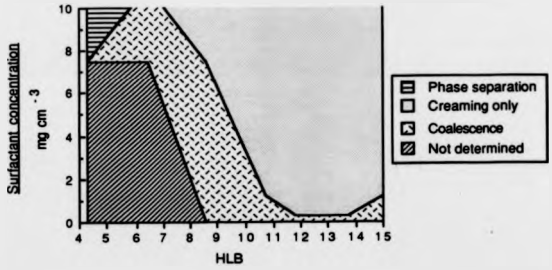
An "HLB scan" can be performed by combining surfactants, one with a low HLB and one with a high HLB, to give a series of HLB values. The ability of the surfactant mixtures to stabilise emulsions can then be assessed visually. Ideally, the total surfactant concentration required to support an emulsion should be minimised to reduce any other effects the surfactant may have on the system. If this process is repeated with different total surfactant concentrations, a surfactant mixture with an optimum HLB and total surfactant concentration can be estimated for any given oil-in-water emulsion system.

Blends of Tween 80 and Span 80 were used in an HLB scan of a diethylphenylmalonate-in-water emulsion system (Figure 3.24). The optimum surfactant mixture had an HLB of between 11.8 and 13.7 at a total surfactant concentration of 0.5 mg $ml^{-1}$. The emulsion was not completely stable over the full time of incubation, as some creaming did occur. However, there was no agitation of the incubation mixtures and it was found that the agitation used in the hydrolysis reaction system abolishes this relatively minor emulsion instability. At low values of HLB, the emulsion was dramatically unstable as coalescence of the oil phase occurred. At very low values of HLB, complete phase separation was observed.

Diethylphenylmalonate is an analogue of 2-phenylpropane-1,3-diol diacetate (3.6) and it can therefore be expected that similar results would be obtained with the latter.

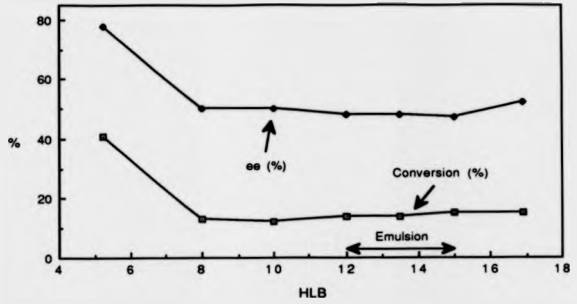
Similar mixtures of C8E1 and Brij 35 and of C8E1 and Tween 80, were used in the PPL-catalysed hydrolysis of the diacetate 3.6. The results are given in Figures 3.25 and 3.26, in which are indicated the HLB ranges over which emulsions were formed. The results indicate no significant effect of emulsification on conversion or ee.

**Figure 3.24** HLB scan of diethylphenylmalonate



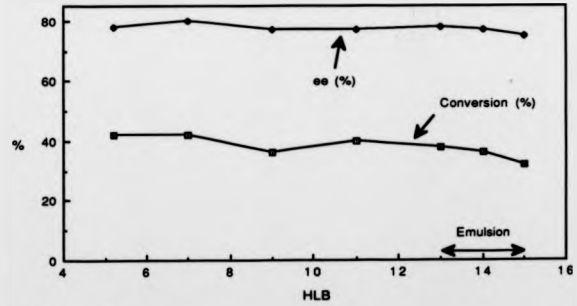

Mixtures of Span 80 and Tween 80 in pH 7.0 phosphate buffer (0.1 mol $dm^{-3}$; 2 $cm^3$) were prepared, giving a series of HLB values and total surfactant concentrations. Diethylphenylmalonate (100 $mm^3$) was added to each mixture and the mixtures were vortexed (1 min) and incubated at 20 °C for 16 h. Creaming, coalescence and complete phase separation were assessed visually.

**Figure 3.25** The effect of the HLB of a mixture of C8E1 and Brij 35 on the PPL-catalysed hydrolysis of the diacetate **3.6**




Reaction mixtures consisted of C8E1 and Brij 35 (2 mg total), pH 7.0 phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), NaCl (0.1 mol $dm^{-3}$), the diacetate **3.6** (10 $mm^3$) and crude PPL (3.75 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 4 h. The formation of a stable emulsion was assessed visually. Conversion and enantioselectivity were determined by chiral HPLC.

**Figure 3.26** The effect of the HLB of a mixture of C8E1 and Tween 80 on the PPL-catalysed hydrolysis of the diacetate **3.6**




See Figure 3.25.

To test further whether the formation of an emulsion had any effect, several other surfactant blends, with an HLB of 14, were used to stabilise emulsions of the diacetate **3.6** (Table 3.11). In all cases the reaction was inhibited regardless of whether or not emulsions were formed by vortexing.

**Table 3.11** The effect of surfactant stabilised substrate emulsification on the PPL-catalysed hydrolysis of the diacetate **3.6**[a]

| Surfactants [w/w (%)] | Emulsified[b] | | Non-emulsified[c] | |
|---|---|---|---|---|
| | Conversion (%)[d] | ee (%)[d] | Conversion (%)[d] | ee (%)[d] |
| None | 35 | 68 | 39 | 76 |
| C8E1 and tauro-deoxycholate (23.5) | 23 | 78 | 16 | 76 |
| C8E1 and Brij 35 (33.3) | 9 | 41 | 8 | 47 |
| C8E1 and Tween 80 (11.1) | 16 | 75 | 14 | 69 |

[a]Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), the diacetate **3.6** (10 $mm^3$), surfactants (0.66 mg $ml^{-1}$ total; HLB 14) and crude PPL (3.75 mg). The mixtures were incubated at 30 °C for 2.5 h.

[b]Vortexed (1 min) prior to the addition of enzyme.

[c]Not vortexed prior to the addition of enzyme.

[d]Determined by chiral HPLC.

To check that the foregoing results were not specific to the chosen diacetate substrate, the effect of PVA acacia stabilised emulsions of 1-phenylethylbutanoate (3.2) was investigated (Table 3.12). There was no significant increase in reaction rate, regardless of whether or not an emulsion was formed.

**Table 3.12** The effect of PVA and acacia stabilised emulsions on the PPL-catalysed hydrolysis of 1-phenylethylbutanoate (3.2)[a]

| PVA (concentration/mg $cm^{-3}$) | Emulsion[b] | Conversion (%)[c] | E[c] |
|---|---|---|---|
| None | no | 36 | 23 |
| Rhodoviol 30/5 (1.5) | no | 36 | 23 |
| Rhodoviol 4/20 (26.7) | yes | 42 | 18 |
| PVA 117 (0.83) | no | 40 | 22 |
| Acacia (100) | no | 28 | 18 |
| None[d] | no | 27 | 12 |
| PVA 205 (5)[d] | yes | 28 | 15 |
| Rhodoviol 25/140 (5)[d] | no | 30 | 13 |
| Rhodoviol 4/125 (5)[d] | no | 35 | 19 |

[a]Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), 1-phenylethylbutanoate (3.2; 10 $mm^3$), surfactant and crude PPL (15 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 4 h.

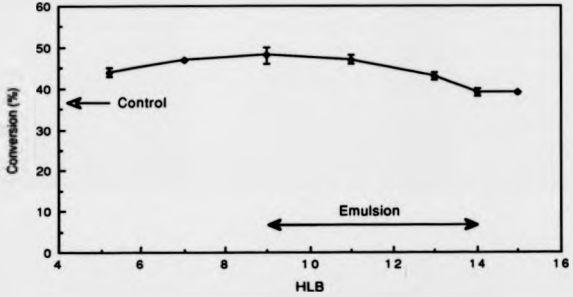
[b]The formation of an emulsion was visually assessed prior to the addition of enzyme and after 4 h.

[c]Determined by chiral HPLC. Conversion was calculated from $ee_s$ and $ee_p$.

[d]Experiments were carried out on a separate occasion.

HLB scans were carried out using 1-phenylethylbutanoate (3.2) with subsequent hydrolysis by PPL. Little or no effect on either the degree of conversion (Figure 3.27) or the enantioselectivity (Figure 3.28) was observed compared with the reaction carried out in the absence of surfactant. In addition, no discontinuity was observed over the range in which emulsions were formed.

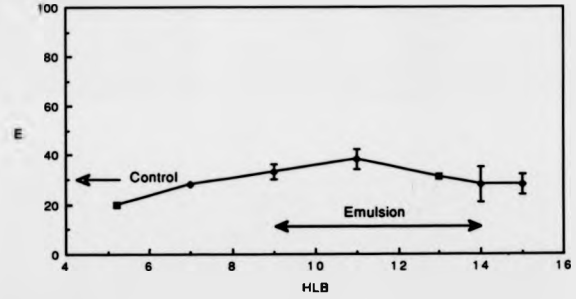
**Figure 3.27** The effect of the HLB of a mixture of C8E1 and Tween 80 on the conversion of the PPL-catalysed hydrolysis of 1-phenylethylbutanoate (3.2)




Reaction mixtures consisted of C8E1 and Tween 80 (2 mg total), pH 7.0 phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), 1-phenylethylbutanoate (3.2; 10 $mm^3$) and crude PPL (15 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 4 h. The formation of a stable emulsion was assessed visually. Conversion and enantioselectivity (Figure 3.28) were determined by chiral HPLC. This experiment was conducted in duplicate.

One of the most potent surfactants in terms of the inhibition of the hydrolysis of 2-phenylpropane-1,3-diol diacetate (**3.6**) by crude PPL was SDS. When hydrolysis of this substrate by crude PPL was studied in the presence of increasing concentrations of SDS (Figure 3.30), the extent of conversion fell steadily to nearly zero, below the CMC of SDS (1 - 2 mmol $dm^{-3}$). However the optical purity of the monoacetate produced fell to a minimum and then rose again to nearly equal the optical purity of the product obtained in the absence of surfactant.

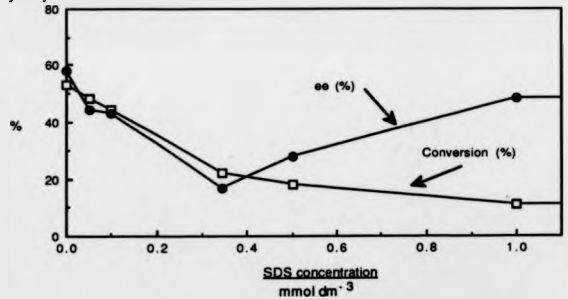
**Figure 3.28** The effect of the HLB of a mixture of C8E1 and Tween 80 on the enantioselectivity of the PPL-catalysed hydrolysis of 1-phenylethylbutanoate (**3.2**)




See Figure 3.27.

Such a result indicates that at least three different enzymatic activities are involved in the hydrolysis of the diester by crude PPL. The results can be interpreted in terms of the differential denaturing action of SDS on the enzymes involved. The main activity, presumed to be the true triacylglycerol acyl hydrolase, is rapidly destroyed so that at *ca* 0.35 mmol $dm^{-3}$ SDS the activity in crude PPL capable of producing predominantly the minor isomer **3.8** is the dominant enzyme. This activity is less susceptible to SDS denaturation than the main activity. With increasing SDS concentration, both these activities decrease to zero, leaving a very minor activity that fortuitously catalyses hydrolysis of the diester with a similar stereoselectivity to that of the main activity.

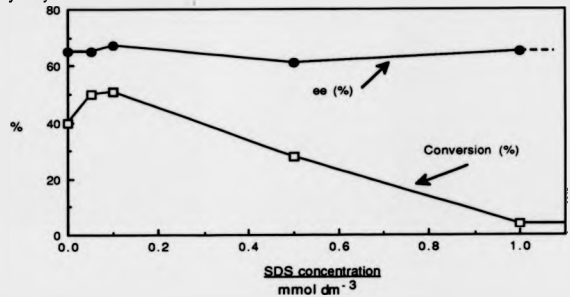
**Figure 3.29** The effect of SDS concentration on the crude PPL-catalysed hydrolysis of the diacetate **3.6**




Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), the diacetate **3.6** (10 $mm^3$), SDS and crude PPL (3.75 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h. Conversion and enantioselectivity were determined by chiral HPLC. The data shown is the average of duplicate experiments.

Support for this interpretation came from a similar study using purified PPL. In this case (Figure 3.30), the ee was constant over the full range of SDS concentrations up to the point where very little activity remained. There also appears to be a rate enhancement on the addition of *ca* 0.1 mmol $dm^{-3}$ SDS. This is considered to be significant as this study was conducted in duplicate.

**Figure 3.30** The effect of SDS concentration on the purified PPL-catalysed hydrolysis of the diacetate **3.6**




Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), the diacetate **3.6** (10 $mm^3$), SDS and purified PPL (Boehringer; 0.1 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h. Conversion and enantioselectivity were determined by chiral HPLC. The data shown is the average of duplicate experiments.

Further supporting evidence came from a comparative study of the effects of several surfactants on the crude and purified enzymes (Figure 3.13). The results demonstrate substantial activity in the crude PPL in the presence of surfactants at concentrations at which the activity of the purified PPL was reduced essentially to zero. In addition, the ee was reduced concomitantly with reduced conversion using the crude enzyme, while the ee remained unchanged with the purified enzyme.

**Table 3.13** A comparison of the effect of surfactants on the crude and pure PPL-catalysed hydrolysis of the diacetate **3.6**[b]

| Surfactant (concentration/mmol $dm^{-3}$) | Crude PPL | | Pure PPL[a] | |
|---|---|---|---|---|
| | Conversion (%)[c] | ee (%)[c] | Conversion (%)[c] | ee (%)[c] |
| None | 53 | 59 | 42 | 63 |
| Brij 35 (0.25) | 26 | 39 | <2 | N.d.[d] |
| LDAO (0.44) | 35 | 48 | <2 | N.d.[d] |
| Taurodeoxycholate (5) | 37 | 44 | 7 | 71 |
| PVA 205 (12.5)[e] | 51 | 60 | 12 | 65 |

[a]Obtained from Boehringer.

[b]Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), the diacetate **3.6** (10 $mm^3$), surfactant and either crude PPL (3.75 mg) or pure[a] PPL (0.1 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h.

[c]Determined by chiral HPLC.

[d]Not determined.

[e]mg $cm^{-3}$.

An alternative method of solubilising the diester **3.6** was explored using a cyclodextrin. It was anticipated that complexation (Figure 3.31) would lead to solubilisation but that the equilibrium concentration of the free diester **3.6** would permit hydrolysis with continuous displacement of the equilibrium leading eventually to complete hydrolysis. In the event, the substrate was effectively solubilised by the cyclodextrin but complexation proved to be so strong that conversion fell to nearly zero at a molar concentration of 2.5:1 (Figure 3.32).

Supporting evidence for the formation of a stable complex was provided by $^1H$ NMR spectroscopy (220 MHz) of a solution of the diester **3.6** and the cyclodextrin (1:1, mol/mol) in $D_2O$. There were small shifts in the aliphatic proton resonances of the diester **3.6**. In the absence of cyclodextrin, the aromatic protons gave a multiplet integrating to 5 protons with a chemical shift of 7.37 ppm. In the presence of cyclodextrin, the aromatic protons gave two multiplets integrating to 2 and 3 protons with chemical shifts of 7.20 and 7.42 ppm, respectively, strongly suggesting the formation of a complex.

Further evidence was obtained when crystals formed spontaneously from the above $D_2O$ solution. The crystals were filtered, washed with $D_2O$ and dissolved in $CDCl_3$. Proton NMR spectroscopy of the resulting solution indicated that the proposed complex dissociated in the organic solvent as the resonances of the diester **3.6** were identical to those obtained in the absence of cyclodextrin. Comparing the integrals of the OMe peaks of the cyclodextrin with those of the OAc peaks of the diester **3.6**, and assuming that the cyclodextrin was pure, the molar ratio of cyclodextrin to diester **3.6** was calculated to be 1.3:1. Considering the fact that the OMe peaks overlapped with other minor peaks of the cyclodextrin, this result is consistent with the formation of a 1:1 complex.

**Figure 3.31** Schematic illustration of the proposed cyclodextrin-2-phenylpropane-1,3-diol diacetate complex

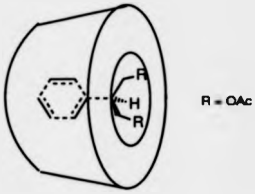
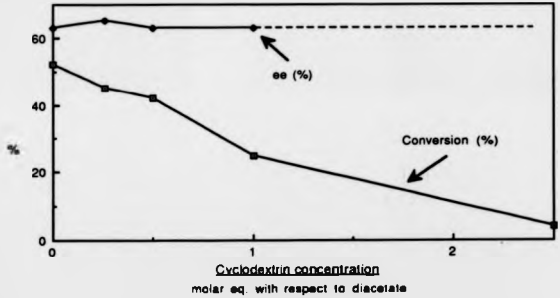


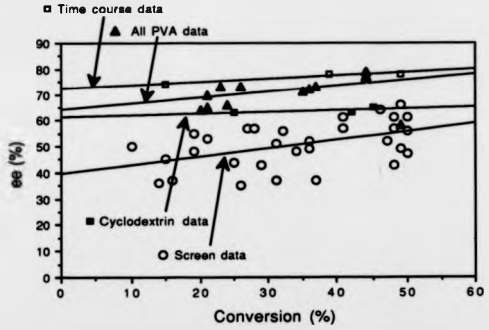

**Figure 3.32** The effect of cyclodextrin concentration on the PPL-catalysed hydrolysis of the diacetate **3.6**




Reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 3 $cm^3$), the diacetate **3.6** (10 $mm^3$), heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin and crude PPL (3.75 mg). The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h. Conversion and enantioselectivity were determined by chiral HPLC. Cyclodextrin concentration was calculated on the assumption that the cyclodextrin preparation was pure.

Consider the data obtained between 0 and 50% conversion from the time course (Figure 3.11), where the ee is essentially constant. A virtually horizontal best-fit line is obtained when these data are plotted as ee against the degree of conversion (Figure 3.33). A similar line is obtained when the data obtained in the presence of the cyclodextrin are plotted. This is not surprising as the sequestration of the diester **3.6** would not affect the enantioselectivity of the reaction. When all of the corresponding data obtained in the presence of PVAs are plotted, a nearly horizontal line is again obtained. However when the data obtained from the screen of surfactants are plotted, there is, in general, a concomitant reduction of ee with a lowering of the degree of conversion.

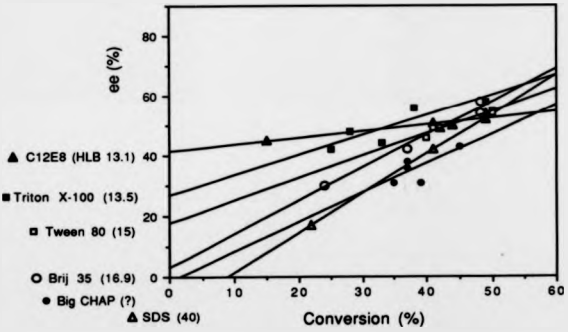
**Figure 3.33** The dependence of the enantioselectivity of the crude PPL-catalysed hydrolysis of the diacetate **3.6** on conversion




The data depicted in Figures 3.11, 3.32 and 3.16, and all data obtained with added PVA are plotted as ee (%) against conversion (%). Only data points where conversion is less than 50% are included. Best-fit lines were calculated using a linear least squares procedure.

If the data obtained with increasing concentrations of surfactants (Figures 3.18, 3.22, 3.20, 3.21, 3.23 and 3.29) are plotted in a similar manner (Figure 3.34), the slopes of the best-fit lines appear to deviate markedly from the horizontal. (Only those data points with conversion between 15 and 50% are included because, as Figure 3.29 shows, very low degrees of conversion can lead to a recovery of the ee to a value obtained in the absence of surfactant.) As expected, the lines intersect at a point near to that obtained in the absence of surfactant.

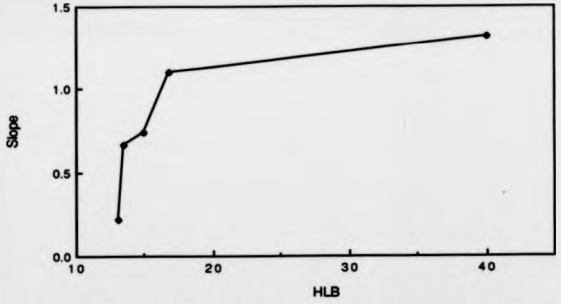
**Figure 3.34** The dependence of the conversion and enantioselectivity of the crude PPL-catalysed hydrolysis of the diacetate **3.6** on surfactant concentration




The data depicted in Figures 3.18, 3.22, 3.20, 3.21, 3.23 and 3.29 are plotted as ee (%) against conversion (%). Only data points where conversion is between 15 and 50% are included. Best-fit lines were calculated using a linear least squares procedure.

A surprising feature of Figure 3.34 is that there appears to be a correlation between the HLB of a surfactant and its ability to reduce the ee of the hydrolysis product. This can be seen more clearly by plotting the slope of the best-fit lines from Figure 3.34 against the HLB of the surfactant (Figure 3.35). From the above correlation, Big CHAP would be expected to have an HLB of between 16.9 and 40. The HLB of Big CHAP has not been reported in the literature. However, considering the fact that Big CHAP is comprised of deoxycholate, which has an HLB of 16, and two sugar residues attached via a linker (Figure 3.1), the overall HLB would indeed be expected to be between 16.9 and 40.

**Figure 3.35** The dependence of the conversion and enantioselectivity of the crude PPL-catalysed hydrolysis of the diacetate **3.6** on the HLB of added surfactants




The slope of the best-fit lines in Figure 3.34 are plotted against the HLB of the surfactant used to obtain each data set.

Finally, a study was carried out into the cholesterol esterase-catalysed[103-105] hydrolysis of the diesters **3.10** and **3.11** of 1,1'-bi-2-naphthol (Figure 3.36). An enantioselective hydrolysis of this substrate has been reported in which PVAs were used in a mixed solvent system of water, ethanol and hexane.[106] The effect of the PVAs in this system was studied with both the butanoyl (**3.10**) and pentanoyl (**3.11**) diesters (Figures 3.37 and 3.38). The results must be interpreted with caution as the complex nature of the reaction makes it difficult to reproduce the results exactly. However, it can be seen that with both substrates there seems to be little or no extra benefit to be obtained by adding PVA over that obtained by the addition of the organic cosolvents alone.

**Figure 3.36** The cholesterol esterase-catalysed resolution of 1,1'-bi-2-naphthol diesters

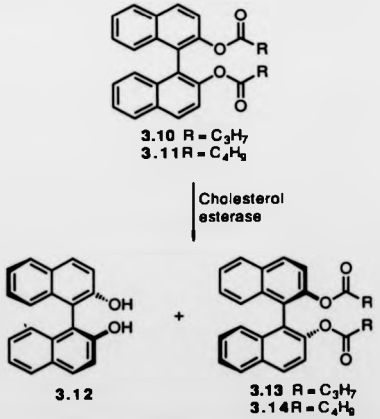
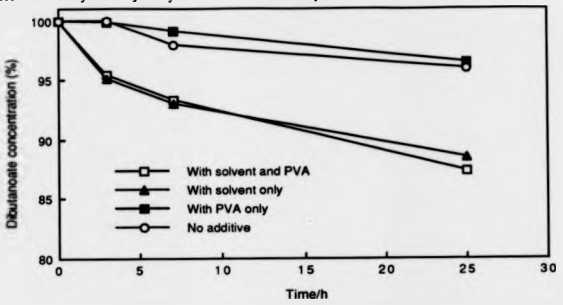
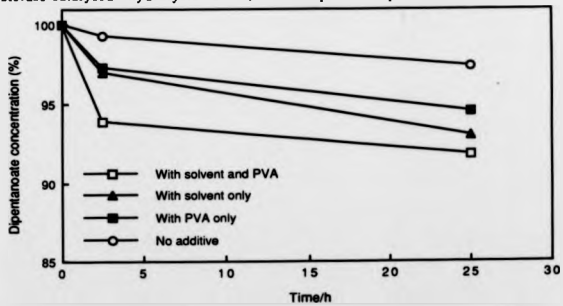


**Figure 3.37** The effect of PVA and organic solvent on the cholesterol esterase-catalysed hydrolysis of 1,1'-bi-2-naphthol dibutanoate (**3.10**)




Reaction mixtures consisted of PVA 117 (5.0 mg), PVA 205 (405 μg) dissolved in $H_2O$ (0.27 $cm^3$), pH 7.0 phosphate buffer (0.1 mol $dm^{-3}$; 2.6 $cm^3$), 1,1'-bi-2-naphthol diester (30 mg), ethanol (0.47 $cm^3$), hexane (0.15 $cm^3$) and cholesterol esterase (2.5 mg). The mixtures were incubated at 30 °C. Control reactions were with PVA and/or organic solvent substituted with $H_2O$. Conversion was determined by HPLC.

**Figure 3.38** The effect of PVA and organic solvent on the cholesterol esterase-catalysed hydrolysis of 1,1'-bi-2-naphthol dipentanoate (**3.11**)




See Figure 3.37.

### 3.3 Discussion

This investigation and previous studies[59,60] have clearly shown that there is no doubt that the rate of hydrolysis of olive oil by PPL is limited by surface area and that the use of some surfactants to stabilise emulsions of olive oil is of benefit. Many researchers have reported the stimulation of triglyceride hydrolysis rates by virtually all types of surfactant although at relatively high concentrations of surfactant, they are all inhibitory.[59,60,73-76] However, the use of surfactants to increase the rate of PPL-catalysed hydrolysis of unnatural substrates has not previously been studied systematically, despite some claims that surfactants are beneficial.[83-86]

It has been shown that the true porcine pancreatic triacylglycerol acyl hydrolase (EC 3.1.1.3) is responsible for the hydrolysis of 2-phenylpropane-1,3-diol diacetate (**3.6**). This has been confirmed in a recent study.[107] In the present investigation, the emulsification of this unnatural substrate by PVAs and mixtures of surfactants, with an optimum HLB, did not lead to an increase in the rate of hydrolysis. Similar results were obtained with the substrate 1-phenylethylbutanoate (**3.2**).

Many of the surfactants used in this study were found to inhibit the rate of reaction significantly. For this reason, it is unlikely that the benefit of stabilising emulsions with surfactants, and increasing the substrate surface area leading to increased reaction rates, would have been realised. This study has revealed that one mechanism of inhibition is very likely to be the denaturation of PPL. This is most certainly the case with SDS, which is known to be one of the most powerful denaturants and can irreversibly denature PPL.[78] This surfactant almost completely inhibits the purified PPL below the CMC. Indeed, all of the surfactants studied, inhibited the crude PPL below their CMC, which is consistent with previous studies.[59,60,73-76] This is not

surprising as surfactant monomers are known to denature water-soluble proteins, which would include PPL, rather than surfactant micelles.[5,55] This explains the finding that non-inhibitory surfactants alleviate the inhibition of PPL by inhibitory surfactants by reducing the total surfactant monomer concentration as a result of the formation of surfactant comicelles, as described by other groups.[55,58,82] Virtually all of the possible effects that surfactants may have on PPL, as discussed below, are a function of surfactant monomer concentration rather than micelle concentration.

An interesting observation was the small but significant in increase in the rate of the pure PPL-catalysed hydrolysis of the diester **3.6** in the presence of *ca* 0.1 mmol $dm^{-3}$ SDS. Similar results have been obtained with water soluble substrates.[108] These findings have been explained by the partial unfolding of PPL by low concentrations of SDS, which mimic the conformational changes accompanying binding to an interface, such that PPL can hydrolyse water insoluble substrates in the absence of an interface. Several-fold rate enhancements were measured with water soluble substrates. This effect of SDS can also be mimicked by sovents such as isopropanol.[109] The small rate enhancement with the water insoluble substrate **3.6** can be explained by PPL being able to hydrolyse the small pool of substrate molecules that are truly dissolved in the aqueous phase in the presence of SDS. This increase in reaction rate was not observed with the crude enzyme, presumable because there are several enzyme activities present in this enzyme preparation.

A recent publication described the noncovalent modification of *Candida cylindracea* lipase by a surfactant, albeit in conjunction with an organic solvent, resulting in the enhancement of the enzyme's enantioselectivity.[110] Surfactants, including SDS, inhibit the purified PPL without any effect on

the enantioselectivity of the enzyme. Similar conclusions were drawn with the enantioselectivity of *Candida cylindracea* and *Pseudomonas* sp. lipases.[85]

However, in this study, there was an effect on the enantioselectivity of the crude PPL. Increasing concentrations of SDS resulted in a loss and then a recovery in the ee of the product. This can only be explained by the presence of three separate enzyme activities in the crude preparation, two with similar and one with poor enantioselectivity, which are subject to differential denaturation by SDS. Differential protein denaturation by SDS is well known in the context of denaturing PAGE and is exploited when SDS is added to proteolytic enzymes in concentrations sufficient to denature selectively the proteins that are targets for proteolysis, while leaving the active protease itself unaffected.[57]

There are several reports of uncharacterised hydrolase activities other than the lipase and cholesterol esterase in porcine pancreas.[100,111-113] The crude enzyme preparation is in no way purified and could contain many esterolytic enzyme activities. One such activity, Lipase C, was found not to be present in the crude material. Distinct activities were not resolved by fractionating the material on a Mono Q anion exchange column with an eluent buffered at pH 8.0.

By contrast, a recent publication[107] described the fractionation of the crude PPL in a similar manner using an eluent buffered at pH 7.5. "Lipase A" (38,000 molecular weight) and "lipase B" (40,000) were resolved using these conditions. Both fractions catalysed the kinetic resolution of the diester 3.6 with high enantioselectivity. It was also confirmed that the authentic acylglycerol acyl hydrolase was active against this substrate. It may therefore be possible that "lipase A" and "lipase B" are the two enantioselective enzyme activities discussed above, and that one of them is

the true acylglycerol acyl hydrolase. These lipases may both be the true acylglycerol acyl hydrolase which differ only in the degree of glycolsylation. Variation in the degree of glycosylation of PPL is known.[59,60] Similarly, isozymes of pig liver esterase have been found to exhibit similar enantioselectivities.[114]

Some surfactants, other than SDS, also appear to reduce the enantioselectivity of the reaction concomitantly with the inhibition of crude PPL. However, others, such as the PVAs which can be inhibitory, do not affect enantioselectivity at all. It is possible PVAs may actually inhibit PPL in a more classical manner because the residual acetyl ester groups present in most PVAs can, in principal, compete with the substrate molecules for the active sites of the esterolytic enzymes. This may also be the case with other surfactants which are themselves possible ester substrates, such as Tween 80, Span 80 and AOT.

Enzyme denaturation and competitive inhibition are probably not the only mechanisms of inhibition. Some nonionic surfactants which are not themselves possible substrates and are not denaturants, such as C12E8 and Triton X-100, inhibit the crude PPL with a loss of enantioselectivity that is not as great as that observed with SDS. It is therefore likely that there is another mechanism of inhibition. It has been suggested from studies with surfactants that inhibit the rate of the PPL-catalysed hydrolysis of triglycerides that lipase molecules are displaced from the interface by surfactants.[60,80] This can occur directly when surfactants compete for the interface with lipase molecules. There is also evidence that surfactants may reduce the interfacial tension to a value below the range in which the lipase can bind efficiently to the interface.[69,70] The displacement of PPL can be reduced by the presence of colipase, which aids the binding of PPL to an interface.[59,60] No benefit could be obtained by the addition of colipase in the system presently

described as colipase is almost certainly present in the crude PPL preparation.[59,60] The reduction of interfacial tension may also lead to an increased incidence of interfacial enzyme denaturation.[4,10,79] Since surfactants were found to inhibit PPL below the CMC, it is unlikely that there was competative binding of the enzyme to the substrate interface and to surfactant micelles.

Finally, an alternative mechanism has been described, whereby surfactants dilute the surface concentration of the substrate.[60] It was concluded that those surfactants with long chain hydrophobic tails are the most potent surface dilutors. It is possible that the rough correlation between the length of the tail of homologous surfactants and the degree of PPL inhibition, in the present investigation, may suggest that surface dilution is indeed a major factor.

It is probable that a combination of all of the above mechanisms was, in fact, the cause of inhibition and that each enzyme activity in crude PPL was affected by these mechanisms differently. Thus the correlation of HLB with the relative effect of surfactants on the enantioselectivity of crude PPL is likely to be fortuitous. SDS is known to be the most powerful denaturant but denaturation is not a function of HLB and denaturation can therefore not be the dominant factor behind this correlation. Similarly there has been no description of a correlation between HLB and either the ability of a surfactant to displace lipases from interfaces or interfacial substrate dilution. Substantial effort would be required, in studies with the pure enzyme, in order to understand which mechanisms were the most dominant. The relative importance of these alternative mechanisms are certainly going to be dependent on the lipase, substrate and surfactant. This illustrates the preferred use of pure enzymes in these studies.

Cyclodextrins have been used to increase rate of the PPL-catalysed hydrolysis of olive oil by forming a complex between the cyclodextrin and the liberated long chain fatty acids, thus reducing product inhibition.[115] In an alternative strategy, it was attempted to disperse the diester **3.6** with a cyclodextrin. The complex that was formed was so stable that the substrate was rendered unavailable for enzymic hydrolysis. The removal of the substrate interface must also be considered because the formation of the complex reduces the volume and surface area of the substrate phase. The formation of complexes between cyclodextrins and small aromatic compounds are well known and several crystal structures of such complexes have been elucidated.[116,117] The use of such complexes for the solubilisation and improved absorption of parenteral drugs is a subject of much interest.[118] The successful application of cyclodextrins for this purpose also requires that the complex is not so stable that the drug bio-availability is effectively reduced to zero.

The results obtained in this study suggest that there is nothing to be gained from the use of surfactants as a means of increasing the rates of reaction in enzyme-catalysed reactions of substrates of low water solubility. The finding, that surfactants of all classes were inhibitory to a greater or lesser extent, is in agreement with the results of other studies.[59,60,77,80,86,119] The effects of two classes of surfactant, PVA and acacia, have been of particular concern, as these substances are used in standard lipase assays against olive oil.[59,60] However, it has been shown that the effect of these surfactants can be emulated by rapid stirring, particularly when an immiscible solvent, isooctane, is added.[68,74,75,86,120-122] PVAs and acacia appear to aid dispersion, making it possible to carry out assays in a smaller volume than when they are omitted. Otherwise, they seem to have no special effect. Indeed, the linearity of the assay system is adversely affected when they are present.[74] The reason why surfactants can be beneficial with long chain

acylglycerols and not with the unnatural substrates is most probably a function of viscosity. The long chain acylglycerols are highly viscous and are therefore more difficult to emulsify.

In the present study of the effect of PVAs on the cholesterol esterase-catalysed hydrolysis of the diesters of 1,1'-bi-2-naphthol (**3.10** and **3.11**), the same conclusion was reached, namely that the addition of PVAs gave no improvement over that achieved by the addition of the cosolvents, hexane and ethanol. Cholesterol esterase is also a surface active lipolytic enzyme which has been shown to be inhibited by some types of surfactant.[59,60,123] In the present study, the use of organic cosolvents was of benefit. Quite the reverse appears to be true with surfactants. However, it would be of interest to systematically study the effect of surfactants on enzymes that function in aqueous solution. It may be that some benefit of the use of surfactants may be realised with esterases and dehydrogenases, for example, as most of the problems associated with surfactants and lipases are interfacial phenomenon which would not affect non-lipolytic enzymes.

Recent developments suggest that the use of ternary solvent systems in biotransformations may prove to be the best strategy in tackling the problem of the low water solubility of substrates.[124-126] Ternary solvent systems are particularly suited to lipase-catalysed reactions because detergentless microemulsions are thermodynamically stable and offer the beneficial effect that surfactants have on increasing surface area without the inhibitory effects of surfactants on lipase activity.

## 3.4 Experimental

**3.4.1** *General Methods.*— Crude porcine pancreatic lipase (PPL; L-3126; Lot 34F-0035), purified PPL (L-0382; Lot 68F-8185), porcine pancreatic cholesterol esterase (C-9530; Lot 25F-96101) and heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (H-0513) were purchased from the Sigma Chemical Co. Ltd. A highly purified PPL preparation was purchased from Boehringer (644072; Lot 115553223-22). Lipase A, B and C were purchased from Enzymatix. Diethylphenylmalonate and 1-phenylethanol were purchased from Fluka. 1,1'-Bi-2-naphthol was purchased from Aldrich. Flash chromatography grade silica gel (mesh 230-400) and TLC plates (Kieselgel 60) were purchased from Merck Ltd. Surfactants were purchased from the suppliers as listed in Table 3.1. All other chemicals, whose sources are not cited, were of the highest quality available. Light petroleum refers to petroleum ether (b.p. 40-60 °C). Free fatty acids were removed from olive oil by chromatography[91] on neutral aluminium oxide as a solution (25%) in diethyl ether and light petroleum (1:10, v/v). Solvent was then removed under reduced pressure. PVAs were dissolved by heating to 80 °C for 1.5 h. Continuous titrations were carried out using a Radiometer ABU-80 Autoburette, TTT-80 Titrator and a TTA-80 stirrer equipped with an 8 mm impeller. The Sonicor SC-152 (150 W) ultrasonic bath was purchased from Orme Scientific. Electrophoresis and protein separations were carried out using the PhastSystem and FPLC equipment, respectively, from Pharmacia LKB Biotechnology. HPLC separations were carried out using a Gilson model 302 solvent delivery system. NMR and IR spectra were obtained using a Perkin Elmer R34 spectrometer and a Perkin Elmer 580B spectrometer, respectively. Mass spectra were determined using a Kratos MS80 spectrometer. UV absorbence was determined using a Unicam SP1800 spectrophotometer. NMR coupling constants (*J*) are cited in Hz. Specific enzymic activity is expressed as nmol of acid liberated per min per mg of protein. Melting points were uncorrected.

**3.4.2** *Protein Determination.*— Protein was determined by the Microprotein Phenol Reagent Method (Sigma procedure No. 690), with blanks containing identical solutions to the test samples except for protein.

**3.4.3** *Titrated Enzymic Hydrolysis of Triglycerides and Unnatural Substrates.*— **3.4.3.1** *Olive oil.* Crude porcine pancreatic lipase (PPL; 18 mg) was added to a mixture consisting of an emulsion of purified olive oil (132 $mm^3$) in a solution (15 $cm^3$) of NaCl (0.1 mol $dm^{-3}$), $CaCl_2$ (0.02 mol $dm^{-3}$) and acacia (10%, w/v). The mixture was stirred rapidly at 23°C and titrated continuously to pH 8.0 with NaOH (0.2 mol $dm^{-3}$). Where indicated, either Rhodoviol 4/125 (0.2 and 2%), Rhodoviol 25/140 (2%) or no surfactant were used instead of acacia.

**3.4.3.2** *Tributyrin.* Crude PPL (0.9 mg) was added to tributyrin (60 $mm^3$) in a solution of NaCl (0.1 mol $dm^{-3}$; 15 $cm^3$). The mixtures were stirred and titrated as described above.

**3.4.3.3** *Unnatural substrates.* Crude PPL (15, 250 and 150 mg, respectively) was added to either 2-phenylpropane-1,3-diol diacetate (**3.6**; 60 $mm^3$), 1-phenylethylacetate (**3.1**; 60 $mm^3$) or 1-phenylethylbutanoate (**3.2**; 60 $mm^3$) in a solution of NaCl (0.1 mol $dm^{-3}$; 15 $cm^3$). The mixtures were stirred and titrated as described above. Reactions were terminated by extraction with diethyl ether (2 × 1 vol.). The combined ether extracts were evaporated under reduced pressure and analysed by chiral HPLC. 2-Phenylpropane-1,3-diol diacetate (**3.6**) was also hydrolysed with cholesteol esterase (1.2 mg), purified PPL (Sigma; 0.5 and 1.6 mg) and purified PPL (Boehringer; 1.0 mg).

**3.4.4** *Fractionation of PPL by FPLC* — Crude PPL (350 mg), added to pH 8.0 Tris.HCl (20 mmol $dm^{-3}$; 10 $cm^3$), was centrifuged (1.8 k × g; 10 min) to remove insoluble material. The supernatant was filtered (0.2 μm) and applied (50 mg.protein) to a Mono Q HR 10/10 anion exchange column, previously

equilibrated in the Tris buffer. The protein was eluted with a NaCl gradient (0 - 1 mol $dm^{-3}$) in the Tris buffer at a flow rate of 3 $cm^3$ $min^{-1}$ and detected by monitoring the absorbence at 280 nm. Fractions (5 $cm^3$) were collected and pooled as indicated in Table 3.5. Aliquots (2 $cm^3$ and 0.2 $cm^3$, respectively) of each pooled fraction were assayed for lipase activity with olive oil and tributyrin, as described above. 2-Phenylpropane-1,3-diol diacetate (**3.6**) hydrolase activity was determined by adding the diacetate (60 m $m^3$) to an aliquot (15 $cm^3$) of each fraction, after adjusting to pH 7.0 with HCl (0.1 mol $dm^{-3}$). The reactions were titrated to pH 7.0 in the manner described previously. The enantioselectivity of the most active pooled fractions were determined by incubating further aliquots (2 $cm^3$) with Tris buffer (1 $cm^3$) containing NaCl (0.1 mol $dm^{-3}$) and the diacetate **3.6** (10 m $m^3$). The reaction mixtures were incubated, extracted and analysed as described above.

**3.4.5** *Buffered Enzymic Hydrolysis of Unnatural Substrates.*— The reaction mixtures consisted of pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 2 $cm^3$) and either 2-phenylpropane-1,3-diol diacetate (**3.6**; 10 $mm^3$) and crude PPL (1 $cm^3$ of a 3.75 mg $cm^{-3}$ solution in phosphate buffer) or 1-phenyl ethanol butanoate (**2**; 10 $mm^3$) and crude PPL (1 $cm^3$ of a 15 mg $cm^{-3}$ solution in phosphate buffer). Surfactant was added where indicated. The mixtures were vortexed (1 min) prior to the addition of enzyme and incubated at 30 °C for 16 h on rotary shakers at 100 r.p.m. Aliquots (0.5 $cm^3$) were removed at the times indicated in the results section, extracted and analysed by chiral HPLC, as described above. Where indicated, purified PPL (Boehringer; 1 $cm^3$ of a 0.1 mg $cm^{-3}$ solution in phosphate buffer) was used instead of crude PPL.

**3.4.6** *HLB Scan of Diethylphenylmalonate.*— Mixtures of Tween 80 and Span 80 (0:10, 2:8, 4:6, 6:4, 7:3, 8:2 and 10:0, w/w) in pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 2 $cm^3$) were prepared giving a series of HLB values (4.3, 6.4, 8.6, 10.7, 11.8, 13.7 and 15, respectively) and total surfactant concentrations

(0, 0.05, 0.5, 2, 5, 10 and 20 mg $cm^{-3}$). Diethylphenylmalonate (100 $mm^3$) was added to each mixture and the mixtures were vortexed (1 min) and incubated at 20 °C for 16 h. Creaming, coalescence and complete phase separation were assessed visually.

**3.4.7** *HLB Scan of 2-Phenylpropane-1,3-Diol Diacetate (3.6) and Subsequent Enzymic Hydrolysis.*— Mixtures of Tween 80 and C8E1 (0:100, 18:82, 39:61, 59:41, 80:20, 90:10 and 100:0, w/w; 2 mg total) in pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$) containing NaCl (0.1 mol $dm^{-3}$; 2 $cm^3$) were prepared giving a series of HLB values (5.2, 7, 9, 11, 13, 14 and 15). Mixtures of Brij 35 and C8E1 (0:100, 24:76, 41:59, 57:43, 69:31, 82:18 and 100:0, w/w; 2 mg total) in phosphate buffer containing NaCl were prepared giving a series of HLB values (5.2, 8, 10, 12, 13.5, 15 and 16.9). 2-Phenylpropane-1,3-diol diacetate (**3.6**; 10 $mm^3$) was added to each mixture and the mixtures were vortexed (1 min) prior to the addition of crude PPL (1 $cm^3$ of a 3.75 mg $cm^{-3}$ solution in phosphate buffer). The reaction mixtures were incubated at 30 °C for 4 h on rotary shakers at 100 r.p.m. The formation of a stable emulsion was assessed visually. Aliquots were extracted and analysed by chiral HPLC as described above.

**3.4.8** *HLB Scan of 1-Phenylethylbutanoate (3.2) and Subsequent Enzymic Hydrolysis.*— Mixtures of Tween 80 and C8E1 in pH 7.0 sodium phosphate buffer were prepared, giving a series of HLB values, as described above. 1-phenylethylbutanoate (**3.2**; 10 $mm^3$) was added to each mixture and the mixtures were vortexed (1 min) prior to the addition of crude PPL (1 $cm^3$ of a 15 mg $cm^{-3}$ solution in phosphate buffer). The reaction mixtures were incubated and analysed as described above. This experiment was conducted in duplicate.

**3.4.9** *Chiral Analysis.*— Substrate and product enantiomers were resolved by HPLC [Chiralcel OB (Baker, Daicel Chemical Industries Ltd.), 25 cm × 4.6 mm]

with an eluent of 2-propanol-hexane. Eluted compounds were detected by UV absorbence at 254 nm. 2-Phenylpropane-1,3-diol diacetate (**3.6**; retention time/min, 16.4), (*S*)-2-phenylpropane-1,3-diol monoacetate (**3.7**; 12.9), (*R*)-2-phenylpropane-1,3-diol monoacetate (**3.8**; 21.5) and 2-phenylpropane-1,3-diol (**3.9**; 7.7) were eluted with 15% 2-propanol at a flow rate of 0.75 $cm^3$ $min^{-1}$. The peaks were asigned by injecting authentic 2-phenylpropane-1,3-diol diacetate (**3.6**), 2-phenylpropane-1,3-diol (**3.9**) and an alkali hydrolysate of 2-phenylpropane-1,3-diol diacetate (**3.6**). The enantiomers of 1-phenylethylbutanoate (**2**; retention time/min, 7.9 and 9.0) and 1-phenylethanol (**3.5**; 11.9 and 16.4) were eluted with 10% 2-propanol at a flow rate of 0.5 $cm^3$ $min^{-1}$. The peaks were asigned by injecting authentic compounds. The extent of enzymic conversion of 1-phenylethylbutanoate (**3.2**) was calculated from $ee_s$ and $ee_p$.

**3.4.10** *Cholesterol Esterase Catalysed Hydrolysis of 1,1'-Bi-2-Naphthol Dibutanoate (3.10) and Dipentanoate (3.11).*— PVA 117 (185 mg) and PVA 205 (15 mg) were dissolved in $H_2O$ (10 $cm^3$) by heating at 80 °C for 1.5 h. The PVA solution (0.27 $cm^3$) and pH 7.0 sodium phosphate buffer (0.1 mol $dm^{-3}$; 1.6 $cm^3$) were added consecutively, with ultrasonic agitation, to 1,1'-bi-2-naphthol diester (30 mg) dissolved in ethanol (0.47 $cm^3$) and hexane (0.15 $cm^3$). The mixtures were vortexed (1 min) prior to the addition of cholesterol esterase (1 $cm^3$ of a 2.5 mg $cm^{-3}$ solution in phosphate buffer) and incubated at 30 °C on rotary shakers at 100 r.p.m. Control reactions with either PVA solution, solvent or both substituted with $H_2O$ were incubated simultaneously. After 3, 7 and 25 h aliquots (0.5 $cm^3$) were extracted with ethyl acetate (1 vol.). The organic extracts were analysed by HPLC (Spherisorb S5 ODS2 (Anachem), 25 cm × 4.6 mm) with an eluent of $H_2O$-MeOH (15:85, v/v). Eluted compounds were detected by UV absorbence at 254 nm. 1,1'-Bi-2-naphthol (**3.12**; retention time/min 3.8), 1,1'-bi-2-naphthol monobutanoate (5.2), 1,1'-bi-2-naphthol dibutanoate (**3.13**; 8.6), 1,1'-bi-2-naphthol monopentanoate

(6.2) and 1,1'-bi-2-naphthol dipentanoate (**3.14**; 13.7) were eluted at a flow rate of 1.0 $cm^3$ $min^{-1}$.

**3.4.11** *2-Phenylpropane-1,3-Diol (3.9).*— A solution of diethylphenylmalonate (4 g, 16.9 mmol) in dry ether (50 $cm^3$) was added to a suspension of $LiAlH_4$ (1.28 g) in dry ether (100 $cm^3$) at 0 °C, with rapid stirring. The reaction mixture was quenched after 2 h with a mixture of powdered $Na_2SO_4$. 10 $H_2O$ and celite (3:1, w/w) at 0 °C, until the precipitate appeared white. The suspension was filtered and the filter cake was washed with ether. The combined organic extracts were dried ($MgSO_4$) and evaporated under reduced pressure to give the crude diol **3.9** (2.46 g). Crystallisation from diethyl ether-light petroleum yielded the diol **3.9** (lit.[127]) (1.41 g, 55%), m.p. 53-54 °C (Found: $M^+$, 152.0832. $C_9H_{12}O_2$ requires M, 152.0837); $\nu_{max}$ ($CHCl_3$)/$cm^{-1}$ 3400, 3050, 2940 and 2880; $\delta_H$ (250 MHz, $CDCl_3$, $SiMe_4$) 2.58 (2 H, s, 2 × OH), 3.08 (1 H, m, CH), 3.95 (4 H, m, 2 × $CH_2$) and 7.30 (5 H, m, Ph); *m/z* (CI, $NH_3$) 170 (M+ $NH_4^+$, 31%), 152 (6), 104 (100) and 91 (16); *m/z* (EI) 152 ($M^+$; 0.5%), 121 (14), 104 (100), 91 (17), 77 (16) and 51 (5); $R_f$ (TLC; ethyl acetate-light petroleum, 1:1, v/v) 0.06.

**3.4.12** *2-Phenylpropane-1,3-Diol Diacetate (3.6).*— The diol **3.9** (1 g, 6.58 mmol) was treated with acetyl chloride (1.03 $cm^3$, 2.2 molar eq.), in dry tetrahydrofuran (25 $cm^3$) in the presence of pyridine (1.06 $cm^3$) and 4-dimethylaminopyridine (1 mg) at ambient temperature, for 16 h. The reaction mixture was quenched with methanol (20 $cm^3$) and evaporated under reduced pressure. The residue was washed with HCl (1 mol $dm^{-3}$) and dissolved in ethyl acetate (20 $cm^3$). The organic phase was washed with HCl (1 mol $dm^{-3}$, 2 × 20 $cm^3$) and saturated $NaHCO_3$ (2 × 20 $cm^3$). The organic phase was dried ($MgSO_4$) and evaporated under reduced pressure. The product oil was applied to a silica gel flash chromatography column and eluted with light petroleum-ethyl acetate (1:1, v/v) to give the diacetate **3.6** (lit.[96,97]) as

a colourless oil (1.36 g, 88%), $\nu_{max}$ (thin film)/cm$^{-1}$ 3020, 2960, 1740 and 1240; $\delta_H$ (250 MHz, $CDCl_3$, $SiMe_4$) 2.02 (6 H, s, 2 × $CH_3$), 3.33 (1 H, m, *J* 6.7, CH), 4.36 (4 H, d, *J* 6.7, 2 × $CH_2$) and 7.32 (5 H, m, Ph); *m/z* (CI, $NH_3$) 254 ($M+NH_4^+$; 13%), 237 ($M+H^+$; 3), 177 (71), 134 (100), 104 (45), 91 (3) and 77 (3); $R_f$ (TLC; ethyl acetate-light petroleum, 1:1, v/v) 0.55.

**3.4.13** *1-Phenylethylacetate (3.1).*— 1-Phenylethanol (**3.5**; 20.26 g, 166 mmol) was acetylated by the method described above, except that the crude product was distilled under reduced pressure (17 Torr; 98-100 °C) to give the acetate **3.1** (lit.[128]) as a colourless oil (19.91 g, 73%), $\delta_H$ (250 MHz, $CDCl_3$, $SiMe_4$) 1.55 (3 H, d, *J* 6.7, $CHCH_3$), 2.08 (3 H, s, $COCH_3$), 5.96 (1 H, q, *J* 6.7, CH) and 7.43 (5 H, m, Ph); $R_f$ (TLC; ethyl acetate-light petroleum, 1:3, v/v) 0.57.

**3.4.14** *1-Phenylethylbutanoate (3.2).*— 1-Phenylethanol **3.5** (16.13 g, 132 mmol) was acylated by the method described above, except that freshly distilled butanoyl chloride was used instead of acetyl chloride. The product oil was applied to a silica gel flash chromatography column and eluted with light petroleum-ethyl acetate (4:1, v/v) to give the butanoate **3.2** as a colourless oil (24.49 g, 96%), (Found: $M^+$, 192.1156. $C_{12}H_{16}O_2$ requires M, 192.1151); $\nu_{max}$ (thin film)/cm$^{-1}$ 3100, 3080, 3050, 2980, 2950, 2890, 1740, 1500, 1460, 1425, 1190 and 1180; $\delta_H$ (250 MHz, $CDCl_3$, $SiMe_4$) 0.93 (3 H, t, *J* 7.8, $CH_2CH_3$), 1.54 (3 H, d, *J* 6.7, $CHCH_3$), 1.67 (2 H, m, *J* 7.8, $CH_2CH_3$), 2.33 (2 H, t, *J* 7.8, $COCH_2$), 5.95 (1 H, q, *J* 6.7, CH) and 7.41 (5 H, m, Ph); *m/z* (EI) 192 ($M^+$; 25%), 105 (100), 104 (75), 77 (31), 71 (32), 51 (10) and 43 (28); $R_f$ (TLC; ethyl acetate-light petroleum, 1:3, v/v) 0.62.

**3.4.15** *1,1'-Bi-2-Naphthol Dibutanoate (3.10).*— 1,1'-Bi-2-naphthol (1.93 g, 6.74 mmol) was acylated with freshly distilled butanoyl chloride as described above, except that the reaction mixture was refluxed for 3 h. Flash chromatography, using light petroleum-ethyl acetate (5:1, v/v) as the eluent,

yielded the dibutanoate **3.10** (lit.[103-105]) as a colourless oil (2.47 g, 86%), (Found: $M^+$, 426.1843. $C_{28}H_{26}O_4$ requires M, 426.1831); $\delta_H$ (250 MHz, $CDCl_3$, $SiMe_4$) 0.57 (6 H, t, *J* 7.5, 2 × $CH_3$), 1.22 (4 H, m, 2 × $CH_2CH_3$), 2.08 (4 H, t, *J* 7.5, 2 × $COCH_2$) and 7.25-7.57 and 7.93-8.08 (12 H, m, Ph); *m/z* (EI) 426 ($M^+$; 3%), 356 (22), 286 (100), 268 (13), 239 (11) and 143 (8); $R_f$ (TLC; ethyl acetate-light petroleum, 1:3, v/v) 0.62.

**3.4.16** *1,1'-Bi-2-Naphthol Dipentanoate (3.11).*— 1,1'-Bi-2-naphthol (2 g, 6.98 mmol) was acylated with pentanoyl chloride as described above, except that the reaction mixture was refluxed for 6.5 h. Flash chromatography, with light petroleum-ethyl acetate (6:1, v/v) as the eluent, gave the dipentanoate **3.11** (lit.[103,105,106]) as a colourless oil (1.44 g, 45%), (Found: $M^+$, 454.2174. $C_{30}H_{30}O_4$ requires M, 454.2144); $\delta_H$ (250 MHz, $CDCl_3$, $SiMe_4$) 0.64 (6 H, t, *J* 5.9, 2 × $CH_3$), 0.91 (4 H, m, 2 × $CH_2CH_3$), 1.11 (4 H, m, 2 × $COCH_2CH_2$), 2.10 (4 H, t, *J* 5.7, 2 × $COCH_2$) and 7.25-7.58 and 7.94-8.10 (12 H, m, Ph); *m/z* (EI) 454 ($M^+$; 6%), 370 (28), 286 (100), 268 (5), 239 (6) and 143 (3); $R_f$ (TLC; ethyl acetate-light petroleum, 1:3, v/v) 0.59. The monobutanoate was also obtained (47%).

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